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THE ROLE OF CALCIUM AND CYCLIC AMP IN VISUAL EXCITATION

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THE ROLE OF CALCIUM AND CYCLIC AMP IN VISUAL EXCITATION

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Aan Ada en mijn ouders

CONTENTS

CHAPTER 1

STRUCTURE AND FUNCTION OF THE ROD PHOTORECEPTOR CELL

1.1. STRUCTURE

1.1.1. Structure of retina and rod outer segments

Light serves in nature as an efficient and important means for information transfer from the environment to the individual organism. In the animal kingdom light is captured in the eye, more specific in the retina, which is part of the nervous system. This thin layer of tissue (approximately 0.1-0.5 mm in thickness) lining the inside of the posterior half of the eyecup, converts light into nervous signals.

Starting from the distal(back) side, the first layer of vertebrate retinal cells, adjacent to the pigment epithelium, is the photoreceptor layer. Here the conversion of light into an electrical impulse takes place. The signal is processed further via a relay layer of neural cells, the bipolar, horizontal and amacrine cells to the most proximal retinal layer, consisting of the ganglion cells (Fig. 1). The axons of the ganglion cells run along the inner margin of the retina and through the optic disc to form the optic nerve. All these cell layers are bound together by neuroglia cells, the Müller cells, which span the full thickness of the retina. As a consequence light has to pass several layers of the vertebrate retina before it reaches the photoreceptors.

Two types of photoreceptor cells can be distinguished in the vertebrate retina: rods and cones, the former generally greatly outnumbering the latter. For instance, in the human retina only about 7% of the photoreceptors are cones. Rods and cones have a complementary function in light reception. The rod has a very high light sensitivity and functions primarily in twilight. In principle it can respond to a single photon (Hecht et al, 1942). It 'perceives' only

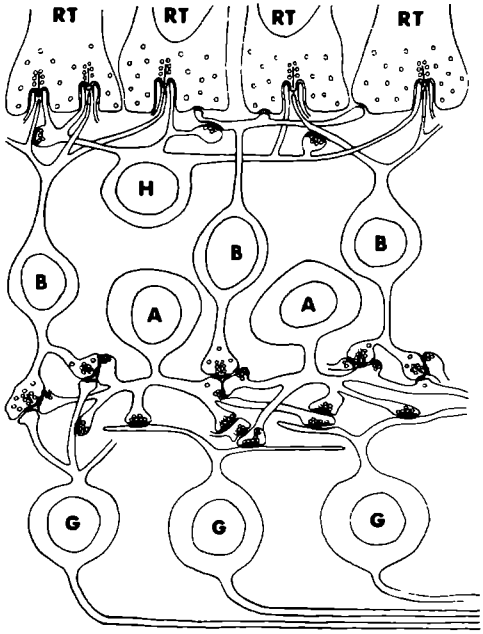


Fig. 1. Schematic diagram showing common retinal synaptic contacts. RT, receptor; H, horizontal cell; B, bipolar cell; A, amacrine cell; G, ganglion cell. This diagram is highly schematic, the cells are not drawn to scale and have been distorted to reveal their more common synaptic contacts. From Dowling (1970).

intensity differences, since a single visual pigment is present in all rod cells. Therefore light capture by rods results in black-white vision. Cones, on the other hand, function mainly under daylight conditions and mediate color-vision. This is due to the existence of three types of cones, each characterized by a single cone pigment with absorption maxima of 575, 535 and 440 nm respectively in man. Thus these three types of cones absorb light in the red, green and blue part of the spectrum. Since much more is known about the molecular mechanism in the vertebrate rod cell than for any other visual receptor cell, due mainly to their abundance and relative ease of isolation and purification, only this photoreceptor type will be discussed in more detail.

The rod cell is an elongated, tubular structure which presents

itself axially to the incident illumination. Morphologically the cell can be divided in an inner segment and an outer segment (Fig. 2). The inner segment contains the usual cell organelles like nucleus, endoplasmic reticulum, Golgi complex and mitochondria. At the synaptic end contact is made with the terminals of horizontal and bipolar cells, while at this level also connections with other photoreceptors occur (Stell, 1972). At the other end the inner segment is linked to the outer segment by a modified ciliary process (cross section $\sim 0.25\mu$), originating somewhere below the apex of the inner segment. The nine pairs of microtubules cross a short ciliary bridge and then continue eccentrially as a sort of 'backbone', running inside the cell membrane of the outer segment. All metabolic processes of the photoreceptor cell

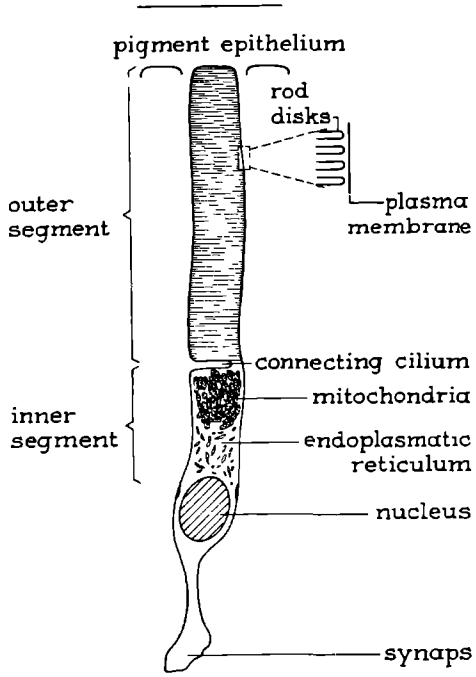


Fig. 2. Schematic diagram of the rod photoreceptor cell.
The arrow shows the direction of the incident light.

are located in the inner segment. The outer segment has no energy supply or biosynthetic apparatus of its own. Energy production and protein synthesis take place in the inner segment and the products are transported to the outer segment through the cilium (Young, 1968). This is possibly the reason why the metabolic active cell organelles (mitochondria, endoplasmic reticulum) lie in the upper part of the inner segment close to the ciliary structure.

The capture of light takes place in the outer segments, which are cylindrical structures with typical dimensions of 50μ x 6μ in frog and 24 μx 1.7μ in rat. They contain a pile of 500 - 2000 flat sacs or discs enclosed by the cell membrane (Fig. 2). In this thesis we shall use the term 'disc'. This does not, however, imply that they are solid structures. On the contrary, there is considerable evidence now for an osmotically active 'intradiscal' space (cf. section 1.1.5). The cell membrane invaginates at the base of the outer segment near the ciliary connection to form a few discs that are open to the extracellular space. All the more distal discs appear to be 'free floating', seemingly without structural connection to the plasma membrane or to each other. There is some evidence for a possible inter-discal matrix of low density material, which could hold the discs together (Cohen, 1968; Falk and Fatt, 1969; Dratz and Schwartz, 1973). The visual pigment, rhodopsin, is located predominantly on the disc membranes. The outer membrane presumably also contains some rhodopsin (Dewey et al, 1969; Hagins and RUppel, 1971; Jan and Revel, 1974), but here it cannot play an important role in light absorption since this membrane contributes only a few percent of the total membrane content of the outer segment. Moreover, only 10% of the outer membrane rhodopsin is in the optimal position for light absorption, i.e. perpendicular to the incident light.

Thus the rod cell is a highly specialized type of cell, uniquely equipped to exercise its function: conversion of electro-magnetic into (electro) chemical energy.

1.1.2. Isolation of rod outer segments

It is relatively simple to isolate intact rod outer segments from dissected retinae. The ciliary connection with the inner segments is very fragile and can easily be broken by means of shaking or mild homogenization. The density of the resulting free rod outer segments (1.08 - 1.10) is low compared to the other cellular fragments like plasma membranes (1.12 - 1.16) and mitochondria (1.14 - 1.18), thus permitting separation by means of flotation on a sucrose cushion (Wald and Brown, 1952; Heller, 1968a) or even better by density gradient centrifugation (McConnell, 1965; Lolley and Hess, 1969; Falk and Fatt, 1973a). Electronmicrographs reveal that the typical structure remains largely intact, although generally the outer segments will be broken once or twice. When a suspension of isolated rod outer segments is treated with a strongly hypotonic solution, they break up into free photoreceptor membrane fragments. An elegant method to assay the average intactness of the plasma membranes of isolated rod outer segments in aqueous suspension is by means of staining with N,N'-didansyl cystine (Ýoshikami et al, 1974).

Of crucial importance is the degree of purity of the preparations, especially in ascribing enzyme activities to the rod outer segments. Light microscopic and electronmicroscopic examinations, while giving an useful indication, cannot unequivocally prove the absence of contaminating structures and do not give quantitative data. Chemical or biochemical analysis can afford a quantitative index of purity. The most reliable parameter for evaluating the quality of a rod outer segment preparation seems to be the rhodopsin content. This protein, which accounts for 80 - 90% of the total protein present can easily be determined quantitatively by spectrophotometry (Hubbard et al, 1971). However, opsin, the photolyzed form of rhodopsin, should be absent. While this is usually the case in outer segments isolated from properly dark adapted laboratory animals, cattle eyes obtained from a slaughter-

house may contain a considerable percentage of opsin (de Grip et al, 1972; Frank et al, 1973). It is, however, possible to convert opsin quantitatively into rhodopsin by addition of 11-cis retinaldehyde, the prosthetic group of rhodopsin, during the isolation procedure (de Grip et al, 1972). A further problem in assessing the purity of outer segment suspensions is the absence of a set of useful marker enzymes characteristic for possible contaminating structures, especially rod inner segment membranes. Finally, the generally used protein determination (Lowry et al, 1951) must be calibrated by amino acid analysis in the presence of rhodopsin (and phospholipids) to give reliable results.

The rod photoreceptor membrane composition has been analyzed extensively. The results of subsequent investigators have gradually changed over the last 20 years as the isolation techniques have yielded purer preparations. At the moment a reasonably consistent picture of the membrane composition is available. On a dry weight basis, 40 - 50% of the membrane consists of lipids, about 80% being phospholipids and only 6% or less cholesterol. Protein also seems to account for 40 - 50% of the dry weight, the bulk (80 - 90%) of which is the visual pigment rhodopsin. The sugars in the membrane have received little attention but they are only present in small amounts (Heller, 1968a; de Grip, 1974). The photoreceptor membranes of cattle and frog, the two species most commonly used, show close similarity in composition (cf. Daemen, 1973).

1.1.3. Rhodopsin

The visual pigment rhodopsin is the main protein component of the rod outer segment membrane. It accounts for approximately 85% of the total membrane protein (Hall et al, 1969; Bownds et al, 1971; Heitzmann, 1972; Robinson et al, 1972; Daemen et al, 1972; Papermaster and Dreyer, 1974). Rhodopsin is a typical membrane protein, completely insoluble in aqueous media, thus requiring the use of detergents for isolation and

purification. Cetyltrimethylammoniumbromide, dodecyltrimethylammonium-bromide (cationic), dodecyldimethylaminoxyde (zwitterionic), Emulphogene BC-720, Triton X-100, Tween 80 and digitonin (non-ionic) have been used to obtain a stable solution in which the spectral properties of rhodopsin are kept intact. Recently the successfull use of the anionic detergent sodium cholate has also been reported (Henselman and Cusanovich, 1974). Conventional column chromatography techniques, adapted for the presence of detergents, have been used in attempts to purify rhodopsin. A major disadvantage is that the replacement of membrane lipids by detergent molecules, i.e. the incorporation of rhodopsin into detergent micelles, induces conformational changes in the protein, resulting in a substantial change in functional parameters. However, recent reports (Hong and Hubbell, 1972, 1973; Chen and Hubbell, 1973; Hong et al, 1973) indicate that the destabilizing effect of the detergent dodecyltrimethylammoniumbromide may be reversed by an exchange with added phospholipids under formation of rhodopsin-containing liposomes, thereby possibly providing a functional rhodopsin system in a well defined micro environment.

Analytical examination of rhodopsin in detergents has been extensive. Molecular weight determinations have been carried out on the basis of amino acid composition, by gel electrophoresis, agarose column chromatography and analytical ultracentrifugation. Although some low values, from 27.000 - 28.600, have been reported for rhodopsin (Shields et al, 1967; Heller, 1968a; Shichi et al, 1969), it now seems well established that the actual value is higher, between 35.000 and 39.000 (Hubbard, 1954; Robinson et al, 1972; Heitzmann, 1972; Daemen et al, 1972; Lewis et al, 1974). Rhodopsin contains a covalently attached carbohydrate moiety (Heller and Lawrence, 1970; Renthal et al, 1973), a feature that has enabled purification by affinity chromatography with concanavalin A (Steinemann and Stryer, 1973).

The characteristic absorption spectrum of rhodopsin solubilized in

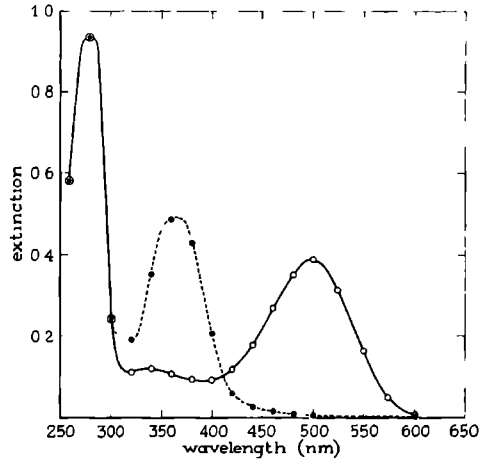
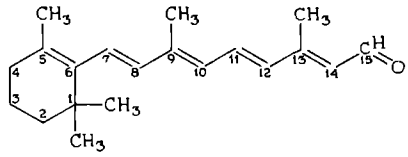


Fig. 3. Absorption spectrum of rod photoreceptor membranes solubilized in 1% digitonin solution, before (solid line) and after (dashed line) illumination in the presence of hydroxylamine.

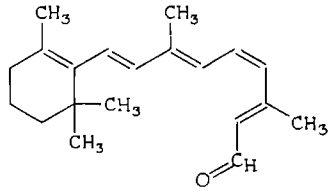
digitonin solution is depicted in Fig. 3. The main absorption bands show peaks at 500 nm(α-band), 340 nm(β-band) and 278 nm(γ-band), the first two representing absorption characteristic for rhodopsin, the last a typical protein absorption band. The α-band and β-band disappear upon illumination, making way for a new absorption band around 380 nm, arising from the liberated chromophoric group. This drop in 500 nm absorbance can be used to determine the amount of rhodopsin present in a given outer segment preparation. The molar absorbance at this wavelength is 40.600 (Wald and Brown, 1953; Daemen et al, 1970). The fact that the spectral sensitivity of the human rod system exactly matches the 500 nm absorption of rhodopsin (Lythgoe, 1937; Chase and Haig, 1938; Wald, 1945; Crescitelli and Dartnall, 1953; Wald and Brown, 1958) provides direct evidence for the involvement of rhodopsin in light perception.

Rhodopsin owes its role in the visual process to the presence of a lipophilic chromophoric group, retinaldehyde (Ball et al, 1948). Only

one of the geometric isomers of retinaldehyde, the sterically hindered 11-cis isomer, is able to combine with opsin to the naturally occurring visual pigment (Hubbard and Wald, 1952a,b). Direct extraction with organic solvents of the chromophore has confirmed that 11-cis retinaldehyde is indeed the chromophoric group of rhodopsin (Rotmans et al, 1972). Only recently, X-ray analysis has revealed the precise structure of this compound (Fig. 4; Gilardi et al, 1971).



all-*trans* retinaldehyde



11-*cis* retinaldehyde

Fig. 4. Structure of the visual chromophores, all-trans (upper figure) and 11-cis (lower figure) retinaldehyde.

The chromophore is linked to an aminogroup in the photoreceptor membrane under formation of a protonated Schiff base (Morton and Pitt, 1957; Rimai et al, 1970). This aldimine bond may in part be responsible for the bathochromic shift of the absorption maximum of 11-cis retinaldehyde upon combination with opsin. In aqueous detergents the maximum absorption of the retinaldehyde lies at 380 nm, while that of the various vertebrate visual pigments derived from it ranges from 440 to 570 nm. Residual color differences can then be explained by secondary interactions between rhodopsin and the π-electron system of the

chromophore (Kropf and Hubbard, 1958). Variations in amino acid composition between different opsins, and thus different interactions with the chromophore, may then lead to the variety in absorption characteristics of rhodopsins from various species (Hubbard and Sperling, 1973).

The aldimine bond which is very labile in model compounds, seems to be reasonably inert in rhodopsin. This suggests that it is buried in the hydrophobic core of the protein (Bownds and Wald, 1965). However, upon illumination the linkage becomes accessible to water, hydroxylamine and borohydride. It has been possible to show that both in native rhodopsin and in the photolytic intermediate metarhodopsin II the chromophore is bound to the ε-amino group of the same lysine residue of opsin (Daemen et al, 1971; Fager et al, 1972; de Grip et al, 1973; Rotmans et al, 1974).

The sole action of light on rhodopsin is a catalytic one: excitation of the 11-cis retinaldehyde chromophore, which isomerizes to the all-trans isomer (Wald, 1968). This photolytic transformation of rhodopsin results eventually in a mixture of opsin and all-trans retinaldehyde. The latter is immediately reduced by the retinol dehydrogenase present in the photoreceptor membrane with NADPH acting as a coenzyme. In <u>vivo</u> the retinol appears to diffuse to the pigment epithelium, where it is stored as the palmitate or stearate ester (Wald, 1935; Krinsky, 1958; Hubbard and Colman, 1959; Dowling, 1960; Hubbard and Dowling, 1962; Futterman and Andrews, 1964; Zimmerman, 1974; Zimmerman et al, 1974). In order to retain visual sensitivity, regeneration of the photolyzed pigment must take place. Evidently, the last step in the regeneration process must be the recombination of opsin with 11-cis retinaldehyde. However, it is not yet known in what form (retinyl ester, retinol, retinaldehyde) and where the chromophoric group is re-isomerized to the 11-cis configuration.

The retinol liberated upon illumination appears to cycle between rod outer segment and pigment epithelium, in a so called long

regeneration cycle (Wald, 1968). However, a short regeneration cycle, located entirely in the rod outer segment, might be functioning as well (Daemen et al, 1974). The occurence of long and short cycles could be regulated by the light intensity. A high light intensity produces large amounts of free retinol, which in view of its lytic properties at higher concentrations must be stored in the pigment epithelium in an inactive form.

1.1.4. Further constituents of the rod outer segments

Other proteins than rhodopsin account for about 15% of the membrane-bound protein. Whether proteins with a mere structural function are present at all remains to be settled. So far no enzymic activity of the rhodopsin molecule per se has been demonstrated. However, a number of claims have been made for the presence of certain enzymic activities, presumably involved in the excitation or regeneration processes, in the rod outer segments.

The presence of a retinol: NADP oxidoreductase seems logical and reasonably well established (Bridges, 1962; Futterman, 1963; de Pont et al, 1970; Kissun et al, 1972). (Na-K)-ATPase (and thus also Mg-ATPase), a typical membrane enzyme, is present in a number of rod outer segment preparations (Bonting et al, 1964; Frank and Goldsmith, 1965; Fedorovich and Ostrovskii, 1968; Etingov et al, 1972; Ostwald and Heller, 1972; Sobota, 1973; Hemminki, 1974). However, the majority of these preparations apparently has not been purified optimally, thus giving rise to contamination especially with (inner segment) cell membranes, which are believed to contain a (Na-K)-ATPase with high specific activity. The purer the outer segment preparation the lower is the specific (Na-K)-ATPase activity (Frank et al, 1973), but some activity appears to be present in the outer segment (this thesis). Recently the presence of enzymes involved in cyclic nucleotide metabolism has been reported. Adenylate cyclase (Bitensky et al, 1971, 1972a,b, 1973a; Miller et al, 1971; Pannbacker, 1974; Miki et al, 1974),

guanylate cyclase (Goridis et al, 1973; Pannbacker, 1973, 1974; Bensinger et al, 1974a,b) and both cAMP- and cGMP-phosphodiesterase (Pannbacker et al, 1972; Bitensky et al, 1973b; Chader et al, 1973; Miki et al, 1973, Pannbacker, 1974; Robb, 1974; Goridis and Virmaux, 1974; Chader et al, 1974a,b,c) are present in purified rod outer segments. Although the claims about extremely high specific activity and light-sensitivity of adenylate cyclase (Bitensky et al, 1971, 1972a; Miller et al, 1971) are superseded (Hendriks et al, 1973; Bownds et al, 1974; Manthorpe and McConnell, 1974), it seems that the presence and involvement in the visual cycle of these enzymes has to be accepted. Finally, recent reports about a light-induced phosphorylation of rhodopsin molecules (Kühn and Dreyer, 1972; Bownds et al, 1972; Kühn et al, 1973; Frank et al, 1973; Bownds et al, 1974; Frank and Bensinger, 1974; Kühn, 1974) make the presence of a protein kinase necessary.

The possible involvement of these enzymes in either excitation or dark adaption will be further discussed in section 1.2.4 and chapters 2, 4 and 7.

The lipid composition of the rod outer segment membranes seems rather unique in that its lipid (40 - 50%) and phospholipid (30 - 40%) percentages are among the highest and the cholesterol content (3%) among the lowest reported for vertebrate membranes (Rouser et al, 1968).

The main phospholipid classes in cattle rod outer segment membranes are phosphatidylcholine (36 - 51%), phosphatidylethanolamine (39 - 43%) and phosphatidylserine (7 - 14%). The phospholipid composition of rat and frog rod outer segments is rather similar (cf. Daemen, 1973). In addition to forming the typical lipid bilayer in the membrane, the phospholipids seem to be of importance for the properties of rhodopsin. Possibly they provide a certain conformation, necessary for the dynamic properties of rhodopsin. While it is possible to delipidate rod outer segments with phospholipase C and remove 95% of the phospholipids without affecting the spectral properties of rhodopsin (Borggreven et

al, 1971), it has also been reported that phospholipids are essential for rhodopsin regeneration (Shichi, 1971; Zorn and Futterman, 1971), in particular the photoisomerization of all-trans retinaldehyde (Shichi and Somers, 1974). Moreover, phospholipids seem to play an important role in the formation of the photolytic intermediate metarhodopsin II (Williams et al, 1974) and in preserving the thermal stability of opsin (Shichi, 1973). These effects may not represent a specific chemical requirement for certain phospholipids, but merely a structural phenomenon. This appears from recent experiments with rhodopsin in model systems, which show regeneration in the absence of phospholipids but in the presence of detergent (Hong and Hubbell, 1973).

A striking phenomenon in all species is the high degree of unsaturation in the fatty acid chains. More than 60% of the fatty acids is unsaturated, the majority even belonging to long-chain polyunsaturated fatty acids as arachidonic (20:4) or docosahexaenoic (22:6) acid (Borggreven et al, 1970; Anderson and Maude, 1970; Nielsen et al, 1970; Poincelot and Abrahamson, 1970; Anderson and Risk, 1974). The fatty acids in phosphatidylcholine are relatively more saturated than those in phosphatidylethanolamine and phosphatidylserine, while in all phospholipid classes the polyunsaturated fatty acid chains are predominantly located at the 2-position of the glycerol moiety (Anderson and Sperling, 1971; Anderson and Risk, 1974). This high degree of unsaturation is apparently of special importance for rod outer segment function. If rats are fed on a diet deficient in essential fatty acids, the retina and the rod outer segments largely retain their original fatty acid pattern, while in most other tissues drastic changes in fatty acid composition occur (Futterman et al, 1971; Anderson and Maude, 1972). When several generations of rats are raised on a fat-free diet, a specific lowering of the docosahexaenoic (22:6) content, accompanied by a significant decrease in amplitude of the electroretinograph a-wave, has been reported (Anderson et al, 1974).

This suggests that this polyunsaturated fatty acid is important for visual function.

1.1.5. Structure of the photoreceptor membrane

Intact rod outer segments are by their regular alignment of thousands of membranes extremely well suited for probing by means of a variety of techniques. Spectroscopy, electronmicroscopy and low-angle X-ray diffraction have given much information about the structure of the disc membrane. Recently also freeze-etching and experiments with spin-labels, chemical modification and osmotic shock have contributed to the present fairly detailed picture of the structure of the rod photoreceptor membrane.

Early electronmicroscopic studies show the photoreceptor membrane to be of a similar triple-layered structure as is generally accepted for most other biological membranes (Hendler, 1971): a lipid bilayer covered on either side by proteins which may penetrate to a greater or lesser extent into the lipid phase. More recently, freeze-etch electronmicroscopy has confirmed these conclusions (Clark and Branton, 1968; Leeson, 1971; Korenbrot et al, 1973).

The regular arrangement of the photoreceptor membranes in the outer segments makes them especially well suited for analysis by means of X-ray diffraction. Fortunately it has been shown that the intensity distribution of the diffraction pattern of rod outer segments in the intact living eye is essentially the same as that in the isolated retina (Webb, 1972). The electron density profiles show a core of low density, corresponding to lipid fatty acid chains, bordered by two regions of higher density, representing proteins and phospholipid-head groups (Blasie and Worthington, 1969; Gras and Worthington, 1969; Blaurock and Wilkins, 1969, 1972; Blasie et al, 1969; Worthington, 1971, 1973; Blasie, 1972; Blaurock, 1972). Estimations of the membrane thickness vary between 55 and 75 Å, the more recent data giving a value of 70 Å. There still exists uncertainty about the shape of the

rhodopsin molecule. While X-ray data seem consistent with the presence of a globular protein, 40 - 50 Å in diameter (Blasie et al, 1969; Blasie, 1972), which partly intrudes into the lipid bilayer, recent energy transfer data employing fluorescent probes suggest an elongated structure with a long axis of about 75 Å (Wu and Stryer, 1972). Such a structure would be able to span the membranewidth. This uncertainty about the shape (and size) of rhodopsin is possibly responsible for the current disagreement on the localization of the molecule (Worthington, 1973).

While some authors advocate that rhodopsin spans the full width of the disc membrane (Chen and Hubbell, 1973; Poo and Cone, 1973; Renthal et al, 1973) calculations of the rhodopsin density in the photoreceptor membrane from various analytical data seem to be compatible with a location of rhodopsin at one side of the membrane (Daemen, 1973). While the possibility that rhodopsin occupies only the side of the membrane facing the intradiscal space is mentioned (Gras and Worthington, 1969; Worthington, 1971, 1973), the view that it is located on the side facing the extradiscal space (Blasie, 1972; Corless, 1972) seems to be more consistent with recent chemical modification data. The use of reagents with different membrane permeability indicates that part of the rhodopsin molecule is exposed to the extradiscal space (Dratz and Schwartz, 1973; de Grip, 1974; Raubach et al, 1974). Moreover, the sugar moiety of rhodopsin appears to be exposed to the aqueous extradiscal environment, as is shown both by optical polarisation (Romhanyi and Molnar, 1974) and binding with concanavalin A (Steinemann and Stryer, 1973; Yariv et al, 1974). Finally, the effect of proteolytic enzymes on photoreceptor membranes suggests that a major part of the rhodopsin molecule is buried in the hydrophobic core of the membrane, while a minor part is exposed to the extradiscal space (Saari, 1974; Trayhurn et al, 1974a,b; v. Breugel et al, to be published). Fig. 5 depicts a schematical drawing of a current view of the disc membrane

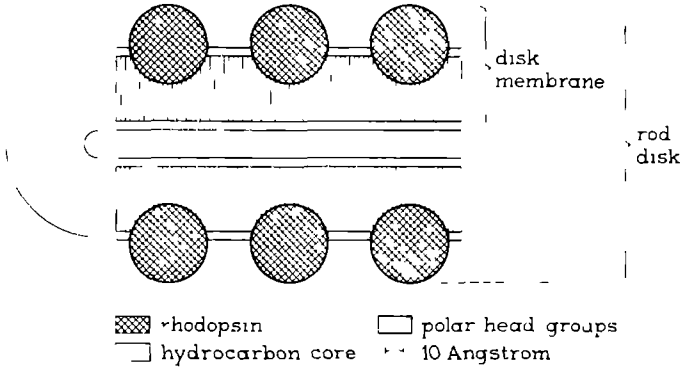


Fig. 5. Schematic model of the frog disc membrane.
After Daemen (1973).

structure. The general dimensions of this model are widely accepted, while uncertainty still exists about the shape of the rhodopsin molecule itself.

The conformation of rhodopsin in the photoreceptor membrane presumably changes upon illumination. It has been concluded from changes in ultraviolet absorption (Hubbard et al, 1965; Ebrey and Honig, 1972), ultraviolet circular dichroism (Crescitelli et al, 1966; Takezaki and Kito, 1967), chemical reactivity (Bownds and Wald, 1965; Falk and Fatt, 1966) and molecular size (Heller, 1968b) of detergent-solubilized rhodopsin that a change in protein conformation accompanies bleaching. Recent additional evidence, obtained from rod outer segment preparations, where rhodopsin is in its native milieu, support this conclusion. Freeze-etching (Mason et al, 1974a), electron spin resonance spectra (Verma et al, 1973) and birefringence measurements (Liebman et al, 1974) indeed suggest that even the structure of the photoreceptor membrane changes upon bleaching of rhodopsin.

The existence of a true intradiscal space is still a matter of some uncertainty. The rather harsh methods of fixation and dehydration,

employed in the preparation of a sample for electronmicroscopy, may very well induce changes from the in vivo situation. This means that the solid disc appearance in most electronmicroscopic pictures may not represent the in vivo situation. Recent reports of electronmicroscopic observations after fixation and dehydration under carefully controlled conditions do indeed point to the existence of such a hydrated compartment between the two disc membranes (Godfrey, 1973; Nir and Pease, 1973; Jones, 1974). Moreover, discs are osmotically responsive to both hypoosmotic (De Robertis and Lasansky, 1961; Brierly et al, 1968; Clark and Branton, 1968; Heller et al, 1971; Falk and Fatt, 1973b) and hyperosmotic shocks (Dowling, 1967; Carless, 1972; Blaurock and Wilkins, 1972). Thus, we can tentatively conclude that a small, osmotically active disc space does appear to exist. This would mean that there are two compartments in the rod outer segment: an intradiscal and an extradiscal space.

The molecular motion and orientation of the pigment molecules have been investigated by means of spectroscopic techniques. Measurements with polarized light (Schmidt, 1938; Denton, 1959; Hagins and Jennings, 1959; Liebman, 1962; Wald et al, 1963) reveal that rod outer segments show linear dichroism with respect to light propagated perpendicularly to its longitudinal axis. This indicates that the chromophoric group of rhodopsin lies parallel to the plane of the disc membrane. End-on illumination (light propagated along the longitudinal axis) of intact rods shows no dichroism. Only with high speed flash photometry a rapidly transient end-on dichroism has been observed (Cone, 1972), which becomes permanent upon fixation with glutaraldehyde (Brown, 1972). This indicates that the rhodopsin molecules rapidly rotate in the plane of the membrane. The rhodopsin molecules also show lateral diffusion in the disc membrane (Poo and Cone, 1973, 1974; Liebman and Entine, 1974). From the rotational relaxation time (20 μsec) and the diffusion constant for the lateral motion (3.5×10^{-9} $cm^2 sec^{-1}$) it has been

calculated that the lipid phase of the disc membrane is highly fluid with a viscosity of about 1P, about equal to that of olive oil. An important factor in determining this high fluidity of the photoreceptor membrane may be the rather highly unsaturated character of the fatty acids in the membrane lipids. For instance, calculations from diffusion constants of phospholipids in sarcoplasmic reticulum vesicles (Scandella et al, 1972), which also show a high degree of unsaturation in their fatty acids, indicate a membrane viscosity of the same order of magnitude. This high fluidity permits a very high velocity of reactions between membrane proteins with an average time between collisions of rhodopsin molecules of about 4 μ sec at 20°C (Poo and Cone, 1974).

Thus the rod photoreceptor membrane presents itself as a rather fluid structure, in which the rhodopsin molecules may be considered to be floating, spinning and drifting, always however maintaining the chromophore in a position optimal for light absorption. While there is no evidence so far to suggest that a close association of rhodopsin with one or a few lipid molecules exists, experiments with enzymic delipidation (Borggreven et al, 1971) do not exclude the possibility of such a relation. Recently developed techniques to incorporate rhodopsin into model membrane systems (Hong and Hubbell, 1972; Chabre et al, 1972; Montal and Korenbrot, 1973) should facilitate the examination of such protein-lipid interactions.

1.1.6. Biosynthesis of rhodopsin and the photoreceptor membrane

The rod photoreceptor membranes have a high turnover rate. Elegant pulse-labeling studies, combining autoradiography and electron-microscopy, show that new discs are continually assembled at the base of each rod outer segment. This occurs at a rate of about one every 40 min for the cold blooded frog to about one every 7 min for warm blooded animals like the monkey (Droz, 1963; Young, 1967; Young and Droz, 1968; Young, 1971a). The disposal phase of this renewal process occurs at the other end of the stack of discs, at the apex of the rod outer segment,

which is enveloped by cytoplasmic extensions of the pigment epithelium. Here groups of discs are intermittently shed by the rods and then immediately phagocytized and degraded by the pigment epithelium (Young and Bok, 1969; Spitznas and Hogan, 1970; Ishikawa and Yamada, 1970; Young, 1971b). Thus a steady state exists in which new membranes are continually formed at one end, while old membranes are digested at the other end of the outer segment.

The components of the photoreceptor membranes are not synthesized in situ. Outer segments are devoid of RNA (Bok, 1970) and incapable of synthesizing protein (Young, 1967; Young and Droz, 1968; Hall et al, 1969). Autoradiographic investigations indicate that protein is transported from the inner to the outer segment by way of the hollow cilium, which connects these two parts of the cell (Young, 1968). Presumably, the other membrane components are delivered by the same route, because it appears that phospholipids are not synthesized de novo in the outer segment membranes. This is indicated both by the absence in the outer segments of the enzymes necessary for phosphatidylcholine synthesis (Swartz and Mitchell, 1970, 1974) and by the failure of outer segments to take up glycerol and incorporate it directly into the membranes (Bibb and Young, 1974b). Therefore, the proteins and the phospholipids which are destined to form the photoreceptor membrane must be produced in the inner segment of the visual cell. It is not yet known, whether all of the membrane constituents remain separate until they are inserted into the growing membrane, or whether some of them may combine prior to membrane assembly (Young, 1974). Experiments with labeled glucosamine show that the glycoprotein is complete at least in terms of its sugar moiety prior to its insertion into the growing disc membrane (Bok et al, 1974).

The site at which the chromophoric group is added to the visual pigment protein is also unknown. Autoradiographic studies reveal that $[^3H]$ retinol is not initially concentrated in the visual inner segment but is bound immediately in the outer segment and becomes even more

concentrated in the pigment epithelium (Young and Bok, 1970). Furthermore, there is no specific localization in the growing discs at the base of the outer segments, whether the animals are kept in the dark or the light (Hall and Bok, 1974). Moreover, incubation of fully bleached retinas in the light does not impede the incorporation of new opsin molecules into growing outer segment membranes (Basinger and Hall, 1973), indicating that opsin can be inserted whether or not retinaldehyde is attached. Thus neither of the two likely alternatives, combination of retinaldehyde and opsin in the inner segment, or addition of retinaldehyde during the initial membrane assembly at the bottom of the outer segment seems probable.

Once the disc membranes have been completely synthesized and are moving upwards in the rod outer segment, movement of rhodopsin molecules is restricted to diffusion inside the disc (Poo and Cone, 1973, 1974). In contrast, rapid exchange of fatty acids between pigment epithelium, outer segment and inner segment appears to take place (Bibb and Young, 1974a), indicating that rods renew their membrane lipids by both molecular replacement and fatty acid exchange.

1.2. FUNCTION

1.2.1. Photolysis of rhodopsin

The sensing of light begins with the absorption of photons by the visual pigment, located in the photoreceptor membranes in the rod outer segments. The one and only action of light is probably the induction of the isomerization of the 11-cis retinaldehyde chromophore (cf. Hagins, 1972). All subsequent thermal reactions (Fig. 6) can proceed in darkness and may be considered as relaxation steps.

Spectroscopy at low temperatures has resulted in the identification of at least six intermediates with different absorption spectra. Their rates of interconversion have been measured. Three of these, prelumirhodopsin (or bathorhodopsin), lumirhodopsin and

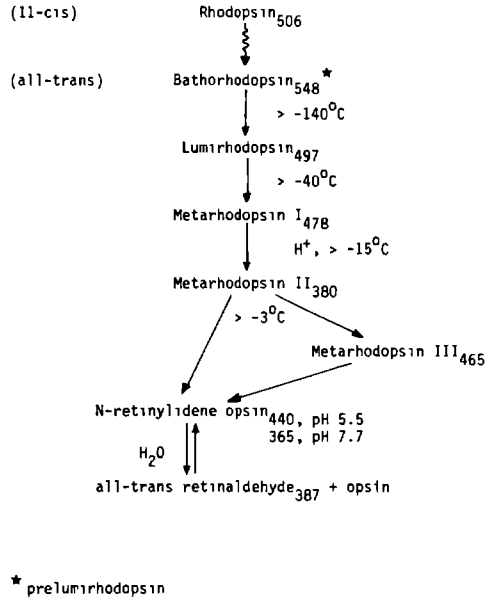


Fig. 6. Intermediate sequence in the photolysis of vertebrate (bovine) rhodopsin in vitro. Arrow with dotted lines denotes the photoreaction, while those with solid lines denote thermal, dark reactions. Subscripts give the absorption maximum (nm) of the corresponding intermediate. The rhodopsin absorption has been measured at -268°C. Adapted from Abrahamson (1973).

metarhodopsin I form and decay within the short latency of visual excitation. The sum of the kinetic delays from the creation of an excited state in a rhodopsin chromophore to the formation of metarhodopsin II at 37°C is about 0.3 msec (Hagins, 1972). Almost all of the delay is due to the relatively long lifetime of metarhodopsin I. Since the disappearance of metarhodopsin II requires several minutes in the living eye (Cone and Cobbs, 1969), this reaction cannot trigger the excitation, because even in the most sluggish animal the latency of vision is usually less than one second.

It seems likely that the conversion of metarhodopsin I to metarhodopsin II is the reaction which triggers the excitatory chain.

The decay of metarhodopsin I has been extensively studied in aqueous digitonin micelles, in intact and sonicated rod outer segments and in situ in the retina (cf. Abrahamson, 1973). There seem to be three reasons to ascribe an important role in visual excitation to this process. First, it is the first step in the photolytic sequence that shows pronounced environment effects: water is required for the reaction and one proton is bound per rhodopsin chromophore (Matthews et al, 1963; Ostroy et al, 1966; Falk and Fatt, 1966; Wong and Ostroy, 1973). Secondly, it appears to correlate temporally with the late receptor potential, which presumably represents excitation of the rod outer membrane (see sections 1.2.2 and 1.2.3). Thirdly, its kinetics show that during the conversion significant macromolecular configurational changes take place. The enthalpy of activation, ΔH^* and the entropy of activation, ΔS^*, which are considered to be empirical measures of the configurational changes during the reaction, indicate a relatively large conformational change compared to the preceding reactions (Abrahamson, 1973). Another indication for a large conformation change is the finding that metarhodopsin II can be reduced by aqueous $NaBH_4$, while metarhodopsin I is not reducible (Bownds and Wald, 1965; Akhtar et al, 1965).

Hence, absorption of a photon by a rhodopsin molecule leads, after isomerization of the chromophore, via a series of thermal relaxation steps to a decay of the metarhodopsin I intermediate, thereby presumably triggering the first step in the excitatory process, which ultimately results in neural excitation.

These primary processes take place in the photoreceptor disc membrane. However, the signal transmission must ultimately result in excitation of the synaps on the proximal end of the rod inner segment. The system that has to span this gap (e.g. 25 - 45μ in rat rods) apparently requires speed as well as high reliability. The three most common physical processes used by cells for internal signal

transmission are (a) diffusion of a substance released upon stimulation, (b) passive polarization of the plasma membrane of the receptor cell generated or controlled by the stimulus, and (c) propagation of action potentials along the cell membrane. The requirements of speed and reliability can be used to assess the contributions of these three processes to the visual excitation mechanism (Hagins, 1972). It appears that both signal transmission by membrane polarization and a transmitter system are involved in the coupling of photon capture to synaptical excitation. Research on light-induced electrical phenomena in the retina has greatly contributed to our understanding of the signal transduction process.

1.2.2. Electrical phenomena

Since the discovery of electrical signs of activity on illumination of the eye, more than a century ago (Holmgren, 1865), much has been done to explain the so-called electroretinogram in terms of visual function. The electroretinogram is usually recorded with electrodes placed on the cornea and the scalp, but it can also be recorded with electrodes placed in the open eye cup on opposite sides of the retina. It may differ in shape according to the species and the state of adaptation, but typically it starts with a cornea-negative deflection, the a-wave, followed by a larger positive potential, the b-wave, and then in the case of a dark-adapted retina a slower positive potential, the c-wave (Fig. 7). The potentials thus recorded consist of the sum of the activities of all retinal cells. Due to the structural complexity of the retina it has been difficult to associate the electroretinogram components with specific anatomical structures. Most generally accepted is the resolution of the electroretinogram into three components, PI, PII and PIII (Granit, 1934; Granit and Riddell, 1934; Fig. 7).

The origin of the c-wave (PI) seems to be well established. It disappears in the rabbit after selective poisoning of the pigment

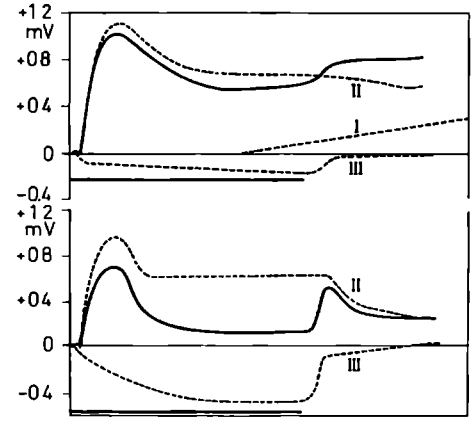


Fig. 7. Analysis of the electroretinogram (frog) into three components (PI, PII and PIII) at dark- (upper) and light-adapted state (lower). From Granit and Riddell (1934).

epithelium by sodium iodate (Noell, 1954) and it is possible to obtain intracellular recordings closely resembling this component from cells of the pigment epithelium in the cat (Steinberg et al, 1970; Schmidt and Steinberg, 1971). The b-wave (PII) is dependent upon the activity of cells in the inner nuclear layer. Clamping the retinal circulation, which supports primarily the ganglion cells and the cells of the inner nuclear layer, completely abolishes the b-wave, leaving however the a-wave (PIII) intact (Brown and Watanabe, 1962a,b). Intraretinal recordings in the cat retina have indicated the bipolar cells as the possible source of the b-wave (Arden and Brown, 1965). More recent work on mudpuppy retina, however, suggests that the b-wave is generated by the glial Müller cells (Miller and Dowling, 1970).

Intraretinal depth recordings in the cat eye in situ (Brown and Wiesel, 1961a,b; Arden and Brown, 1965) and in the isolated rabbit retina (Hanitzsch and Trifonow, 1968) show that the a-wave (PIII) is localized in the receptor layer. This conclusion is confirmed by the previously mentioned experiments in which the retinal blood circulation is selectively clamped. The clamping of the retinal circulation, which

extends only to the outer margin of the inner nuclear layer, leaves the a-wave intact. This is presumably due to the fact that the receptor layer is supplied primarily by the choroidal circulation (Brown and Watanabe, 1962a,b). Thus in mammals the a-wave seems to be generated solely in the photoreceptors and therefore can be referred to as the receptor potential. However, in cold blooded retinas a somewhat different situation apparently exists. Fractional recordings of the frog and carp electroretinogram (Murakami and Kaneko, 1966; Murakami and Sasaki, 1968a,b) indicate that the a-wave is of dual origin. It is composed of a distal component originating from the receptors and a more proximal component arising from structures other than the receptors, possibly the MÜller cells (Faber, 1969). While this receptor potential appears to have an ionic origin and follows the conversion of metarhodopsin I, there is another earlier electrical phenomenon of non-ionic character.

Flash illumination of the retina results in a fast transient potential change (Brown and Murakami, 1964a,b; Cone, 1964; Pak and Cone, 1964; Brindley and Gardner-Medwin, 1966; Cone and Cobbs, 1969) which has a latency of less than 20 μsec and is very weak compared to the a-wave. This fast photovoltage is called the 'early' receptor potential (ERP) to distinguish it from the a-wave or 'late' receptor potential. Both receptor potentials seem to be generated by different processes. Ion fluxes across cell membranes, which presumably generate the a-wave, apparently cannot be the source for the early receptor potential, since this voltage persists under conditions in which the normal ionic batteries of cells are likely to be discharged. It appears that this potential change can reasonably be attributed to a change either in the orientation or magnitude (or both) of the electric dipole of the rhodopsin molecule.

The charge displacements which generate the early receptor potential appear to be much too small to act as excitatory electrical signals transmitted to the synaptic end of the receptor, except

possibly at very high light intensities (Hagins and Rüppel, 1971). Moreover, as the sites of the charge displacements seem to be the plasma membranes (Hagins and McGaughy, 1968; Rüppel and Hagins, 1973) or possibly the disc membranes at the base of the rod outer segments where they still are continuous with the outer membrane (Falk and Fatt, 1972), it seems improbable that the early receptor potential, at least in vertebrates, has a causative role in visual excitation.

1.2.3. Dark current and photocurrent

The light-evoked electrical response of photoreceptors has been extensively studied. Different approaches have been used to separate the receptor response from the responses from other retinal cells. The most direct way is, of course, to make intracellular recordings from isolated photoreceptor cells.

The first intracellular recordings have been made from the large photoreceptor cells of the mudpuppy (Bortoff, 1964; Bortoff and Norton, 1965a,b). Later work on the same material (Werblin and Dowling, 1969; Toyoda et al, 1969) indicates that here the identification of the cell types has not been unequivocal. This identification of cells from which recordings are made, is of course particularly important in assigning specific responses to single cell types. The best method of identification is by means of electrode marking, i.e. by injection of a dye through the inserted multiple microelectrodes (Kaneko and Hashimoto, 1967; Stretton and Kravitz, 1968). This technique of cell identification, combined with a very elegant technique to insert a pipette into the cell by jolting the retina at a high acceleration towards a vertically held, slowly advancing micropipette (Tomita and Kaneko, 1965) has yielded satisfactory intracellular recordings from carp (Tomita et al, 1967) and mudpuppy (Toyoda et al, 1969) photoreceptors. These recordings, which presumably represent cone responses, have been complemented by intracellular recordings from gecko (Toyoda et al, 1969) and frog (Toyoda et al, 1970), representing

predominantly rod responses. All recordings from vertebrate retinas show the same behaviour: a relatively small resting potential (inside negative) and a hyperpolorization upon illumination in both rods and cones (Tomita, 1970). This hyperpolarization seems to be accompanied by an increase in the resistance of the outer membrane (Toyoda et al, 1969; Baylor and Fuortes, 1970). The increase in membrane resistance is not a secondary effect of the hyperpolarization (Bortoff and Norton, 1967), since a hyperpolarizing extrinsic current has no effect upon the membrane resistance (Toyoda et al, 1969; Baylor and Fuortes, 1970). Thus the increase in membrane resistance appears to be a phenomenon directly related to the membrane process which causes the hyperpolarization. It should be mentioned that some recent experiments fail to confirm a decrease in membrane conductance (Falk and Fatt, 1973a; Lasansky and Marchiafava, 1974).

The photoreceptor response has also been measured by means of extracellular recordings. In this case the rod response is isolated either by careful insertion of two or three microelectrodes at different levels in the receptor layer (Penn and Hagins, 1969, 1972; Hagins et al, 1970; Ernst and Jagger, 1973; Ernst et al, 1974) or by suppressing the other components of the electroretinogram with sodium aspartate (Sillman et al, 1969; Zuckerman, 1971, 1973) or removal of calcium ions from the medium (Arden and Ernst, 1969a,b). From these experiments it is concluded that there runs along each dark adapted photoreceptor a steady 'dark current', which leaves the inner segment and enters the outer segment (Penn and Hagins, 1969; Hagins et al, 1970; Zuckerman, 1973). Illumination produces a reduction in the dark current, which has the same time course as the a-wave of the electroretinogram. This reduction in the dark current is often described as a light-induced photovoltage. The amplitude of this photovoltage increases with the light energy up to a certain level. The maximum response amplitude is that just sufficient to cancel the dark current (Penn and Hagins,

1972). The fact that local illumination of the outer segments produces a relatively local photocurrent (Hagins et al, 1970) shows that it is in fact a primary electrical effect and not a feedback from neurons more proximal to the brain.

Thus photolysis modulates the dark current. This decrease in dark current then results in synaptic stimulation. It can be calculated that the photocurrent produced by rat rod outer segment is large enough to explain its ability to 'see' a single absorbed photon (Hagins et al, 1970).

The only mechanism of charge transport responsible for the dark current which can be seriously considered, appears to be ion movement. Identification of the charge carriers has been achieved by changing the ionic environment of the cells. There have been several studies of the effects of various ions on the electroretinogram (Furukawa and Hanawa, 1955; Hamasaki, 1963; Hanawa et al, 1967), but their interpretation is difficult because other cells than the receptors contribute to the electroretinogram. Intracellularly applied micropipettes are easily dislodged during change of the chemical environment. Hence, the significant studies have been those where the effect of ionic changes on the isolated receptor responses has been determined with extracellularly applied microelectrodes.

Removal of sodium or addition of ouabain rapidly inhibit the dark current, indicating that sodium is the charge carrier. Observations on the PIII component persisting after aspartate treatment of the retina (Sillman et al, 1969) and on the directly measured receptor currents of rod outer segments (Yoshikami and Hagins, 1970, 1973; Zuckerman, 1971, 1973), as well as intracellular recordings of rod responses (Brown and Pinto, 1974) are in general agreement with this conclusion. The amplitude of the photoresponse decreases with increasing potassium concentration and is independent of changes in the external chloride concentration (Sillman et al, 1969; Winkler, 1972). Earlier

conflicting results from rat retinas (Arden and Ernst, 1969a,b) are possibly, at least in part, due to the fact that the receptor potential is isolated by removing calcium ions from the medium, thus making comparison with responses recorded in conventional Ringer solutions difficult. Later results from the same authors (Arden and Ernst, 1972) can possibly be explained by the fact that generally variable non-receptor sinks and sources of current exist in the receptor layer (Ernst et al, 1974).

Nothing definite is known about the distribution of sodium and potassium in the vertebrate rod outer segments. This is mainly due to the fact that the extended isolation methods may alter the ionic composition. In addition, rod outer segments undergo structural, and presumably functional, changes shortly after being separated from the retina (Robertson, 1966). In fact it has been reported that during isolation of cattle rod outer segments by means of a conventional procedure using gradient centrifugation, 75% of the original sodium and potassium is lost (Etingof et al, 1970). The large divergence in characteristics of the outer segment preparations used and in methodological approaches, is probably the cause of the many conflicting results obtained in earlier studies (cf. Etingof, 1972). These studies of changes in ion content or of tracer fluxes upon illumination of isolated rod outer segments apparently cannot be trusted. More promising in this light are the elegant experiments where the permeability characteristics of frog rod outer segments are determined by observing their osmotic behaviour (Korenbrot and Cone, 1972; Korenbrot, 1973; Bownds et al, 1973, 1974; Korenbrot et al, 1973). This technique involves minimal manipulation of the outer segments and measurements can be completed within minutes after separation from the retina. These experiments clearly show that dark-adapted outer segments are permeable for sodium and impermeable for potassium, while illumination produces a specific decrease in the sodium permeability of

the outer membrane. This phenomenon has been confirmed in X-ray experiments with intact frog retina (Chabre and Cavaggioni, 1973).

Many of the characteristics that have been found in different types of experiments can be accounted for in the following model for the rod photoreceptor. The rod contains an ouabain-sensitive sodium pump, located in the inner segment, which pumps sodium ions into the space between adjacent photoreceptor cells. This pump, which is sensitive to cyanide, is driven by mitochondrial oxidative phosphorylation. It can be either a neutral or an electrogenic pump. The extruded sodium ions flow along the rod cell and enter the outer segment, which in the dark adapted state has a high sodium permeability. Thus positive charge will flow continuously from the synaptic end to the outer segment through the extracellular space and in the reverse direction intracellularly. This ion current seems to keep the synapse activated. The latter presumably responds by continuously releasing a transmitter substance (cf. Lam, 1972; Dowling and Ripps, 1973), which keeps the connected horizontal and bipolar cells depolarized (Dowling and Ripps, 1973). During illumination the sodium current will be decreased by the reduction of the sodium permeability of the outer segment membrane (Fig. 8). The membrane will then hyperpolarize, since the membrane potential will become dominated by the potassium permeability. This leads then to a decrease in transmitter release and consequently to a hyperpolarization of horizontal and bipolar cells. It can be calculated that the absorption of one photon and thus the excitation of a single molecule of rhodopsin, briefly reduces the sodium-influx by about 3% (Penn and Hagins, 1972), preventing some 10^7 sodium ions from flowing into the outer segments (Korenbrot and Cone, 1972). This current gain, the number of electronic charges flowing (or better: prevented from flowing) per absorbed photon, exceeds the theoretical requirement for synaptic excitation by several orders of magnitude (Hagins, 1972). This way, the question how the energy of one photon suffices to stimulate the synaps is resolved too. The metabolic energy of the ATP-driven sodium pump can be used, although indirectly.

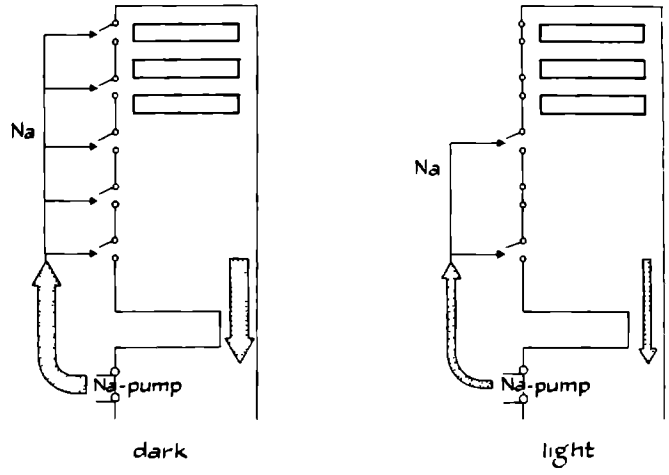


Fig. 8. Sodium currents in visual excitation

In this way excitation of the rhodopsin molecule, by changing the conductance of the outer membrane, ultimately leads to synaptic stimulation. This clearly poses an important problem. How can excitation of a molecule rhodopsin, taking place on the disc membrane, result in the closing of sodium channels in the rod outer segment outer membrane ? Some form of transmission is needed to bridge the gap between discs and outer membrane.

1.2.4. Signal transmission between disc and outer membrane

The communication between disc and outer membrane apparently must be by one (or a combination) of the processes mentioned in section 1.2.1. There is, however, no evidence for the existence of action potentials. Membrane polarization seems improbable too, since there exists no structural connection between both membranes. Moreover, the early receptor potential, which is caused by changes in the electrical dipole of the rhodopsin molecules, apparently involves only those relatively few molecules, which are indeed directly connected with the outer membrane (see section 1.2.2).

Thus it appears necessary to postulate the existence of a transmitter which somehow links the excitation of rhodopsin to the

change in sodium permeability of the outer membrane. In view of the existing evidence, two possibilities should be seriously considered: a process which involves cyclic nucleotides or a release from discs of some ionic species, presumably calcium ions, which are able to close the sodium channels. It might not even be altogether impossible that both cyclic nucleotides and calcium are involved in excitation.

Cyclic adenosine 3',5'-monophosphate (cAMP) mediates a number of specialized cellular functions (Jost and Rickenberg, 1971). It is known to influence sodium transport through cell membranes (Watlington, 1969; Civan, 1970) and therefore could be involved in decreasing the sodium permeability of the outer membrane. The intracellular concentration of cAMP is a function of the rate of its production by the enzyme adenylate cyclase, and the rate of its hydrolysis by the enzyme cyclic nucleotide phosphodiesterase. Both enzymes are reported to be present in the rod outer segments. The extremely high adenylate cyclase activity, claimed in earlier reports (Bitensky et al, 1971, 1972; Miller et al, 1972) has been shown to be in fact a 100-fold too high (see chapter 2) and this activity could possibly originate from contaminating retinal material. Nevertheless, the presence of a cAMP forming enzyme has now been reported in the rod outer segments from frog (Bitensky et al, 1973; Miki et al, 1973, 1974), mouse (Lolley et al, 1974), cattle (Pannbacker, 1973, 1974; Chader et al, 1973) and man (Pannbacker, 1974). Furthermore, a cAMP phosphodiesterase with a specific activity much higher than the cyclase, is shown to be present in the same mammalian photoreceptors (Miller et al, 1971; Pannbacker et al, 1972; Dumler and Etingof, 1973; Schmidt and Lolley, 1973; Miki et al, 1973; Pannbacker, 1974; Chader et al, 1974a,b,c).

Involvement of cAMP in visual excitation would require regulation of its intracellular concentration by light. A light-dependent enzyme activity is indeed reported to exist in rod outer segments. The level of cAMP seems to be regulated by a light-stimulated phosphodiesterase (Chader et al, 1973, 1974a; Miki et al, 1973), which can also explain

the earlier claimed decrease in adenylate cyclase activity upon illumination (Chader et al, 1973; Miki et al, 1973, 1974).

The effects produced by cAMP in various types of cells are postulated to result from phosphorylation of certain cellular proteins, mediated by the action of cAMP on protein kinases (Kuo and Greengard, 1969). The presence of a cAMP dependent protein kinase in rods has indeed been reported (Johnson et al, 1971; Bitensky et al, 1972b; Pannbacker, 1974), but the light-dependent phosphorylation of rhodopsin apparently is independent of cAMP (Frank et al, 1973; Frank and Bensinger, 1974).

Evidence has accumulated recently that cyclic guanosine 3',5'-monophosphate (cGMP) has at least as specific a role in neural tissue as that ascribed to cAMP and that it does not merely mimic the effects of cAMP (Kuo et al, 1972; Lee at al, 1972). Guanylate cyclase also appears to be present in rod outer segments (Pannbacker, 1973, 1974; Bitensky et al, 1973; Goridis et al, 1973; Bensinger et al, 1974), while the cyclic nucleotide phosphodiesterase shows a clear preference for cGMP as a substrate compared to cAMP (Miki et al, 1973; Pannbacker, 1974; Goridis and Virmaux, 1974; Chader et al, 1974a,c). The evidence for regulation by light is contradictory. Guanylate cyclase is reported to be insensitive to illumination (Goridis et al, 1973), inhibited by illumination (Bensinger et al, 1974) and inhibited only after a certain time lag (Pannbacker, 1973), while the phosphodiesterase too has been described as being insensitive (Goridis and Virmaux, 1974) and sensitive (Chader et al, 1974a) to light.

Taking all evidence together, the involvement of cyclic nucleotides in photoreception is certainly indicated. However, a role in visual excitation seems doubtful (see also chapter 2). Apart from the uncertain relation between bleaching of rhodopsin and enzyme activity, there exists no physiological proof for such a transmitter function. Moreover, the activation or inhibition of one or more enzymes might make

the fluctuation of the cyclic nucleotide levels too slow to act as a transmitter in the excitation process. A role for cyclic nucleotides in the regulation of rod sensitivity seems more likely.

The time constraints posed upon the mechanism of signal transmission (from disc to outer membrane) by the short latency between photon capture and appearance of the photocurrent, seems no restrictive factor in the hypothesis which assignes the transmitter role to calcium (Yoshikami and Hagins, 1971; Hagins, 1972). Three assumptions have been made in order to make this postulated process feasible (Fig. 9):

- *The concentration of calcium ions is maintained by pumps at a much* lower level in the outer segment cytoplasm than inside the discs and in the external solution;
- The sodium conductance of the rod envelope membrane decreases as the cytoplasmic calcium concentration increases, possibly because internal

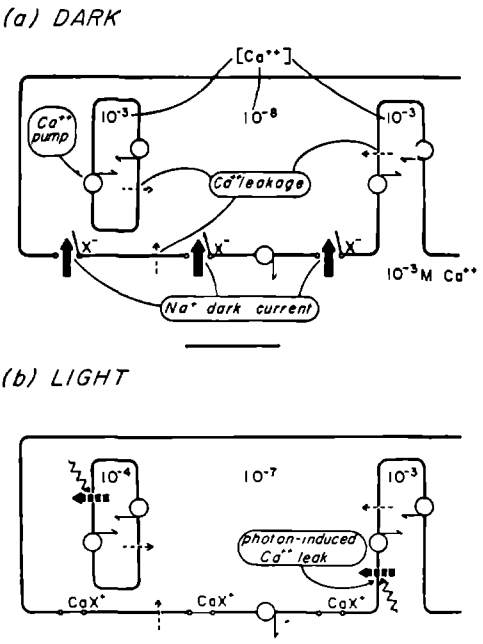


Fig. 9. Model for excitation in vertebrate rods (and cones). The values for calcium ion activities in cytoplasm and discs are estimates derived from studies of nerve and muscle and do not represent actual measurements. After Hagins (1972).

calcium reversibly blocks the sodium channels;

- Light transiently increases the permeability of the disc membranes to calcium, allowing many more than one calcium ion per photon absorbed to enter the cytoplasmic space. Theoretical arguments indicate that one absorbed photon should release at least 100 calcium ions (Cone, 1973; Yoshikami and Hagins, 1973).

This model seems attractive because it attributes to calcium a transmitter function it is already known to possess in muscle and presynaptic nerve endings (Katz, 1966). Moreover, no special assumptions are necessary to distinguish rods from cones, since in cones the calcium ions could enter from the extracellular space.

The hypothesis is based on electrophysiological evidence, in particular on the effects of changes in the extracellular calcium concentration on dark- and photocurrent. Calcium mimics the effect of light: increasing the extracellular calcium concentration decreases the dark current and illumination thereupon fails to elict a photoresponse. Lowering the calcium concentration temporarily increases both dark current and photocurrent, but eventually desensitizes the rod (Yoshikami and Hagins, 1973; Snyder, 1974; Winkler, 1974; Brown and Pinto, 1974). These effects can be explained by assuming that calcium regulates the sodium permeability of the cell membrane and thus the membrane potential. As an increase in the extracellular calcium concentration presumably enhances the cytoplasmic calcium concentration, it can be postulated that the cytoplasmic calcium in fact determines the sodium permeability of the cell membrane. This seems to be confirmed by recent experiments using the ionophore X537A (Hagins and Yoshikami, 1974).

No direct evidence for the calcium-coupling hypothesis exists. Rod outer segments appear to contain a fairly large amount of calcium (Liebman, 1974; Hendriks et al, 1974; this thesis) and experiments using radioactive calcium show an ATP-dependent uptake of this ion

(Bownds et al, 1971; Neufeld et al, 1972; Mason et al, 1974b). Furthermore, both light-induced changes in electrical impedance (Falk and Fatt, 1973a) and volume (Heller er al, 1970) are interpreted to arise from a permeability increase in the disc membrane. However, no light-induced calcium movements have been satisfactorily demonstrated to exist. More questions arise which need to be answered, e.g.: How is the calcium present in the discs, is it really inside the intradiscal space or is it bound to the disc membrane, possibly on the outside ? How can the isomerization of the rhodopsin chromophore lead to release of the calcium ions ? What happens during dark adaptation, how is the original state restored ? Therefore, much more is to be known to confirm the validity of the model, which is the basis of a large part of the work described in this thesis.

CHAPTER 2

ADENYLATE CYCLASE AND ROD PHOTORECEPTOR MEMBRANES

2.1. INTRODUCTION

The hypothesis that cAMP might play a role in visual excitation is a fairly recent one (Bitensky et al, 1971, 1972a,b; Miller et al, 1971). Ever since adenylate cyclase and cAMP were detected and characterized (Rall et al, 1957), the evidence for its participation in a large number of cellular processes has been growing (Jost and Rickenberg, 1971). The cyclic nucleotide mediates the effects of a variety of hormones and other biologically active agents by acting as a second messenger (Robinson et al, 1968). In this case these hormones and other regulatory molecules exert their effect by a stimulation of the membrane-bound adenylate cyclase, thus communicating the signals from the extracellular space to intracellular enzyme systems. Cyclic AMP can also influence the transport of ions through cell membranes. It has been shown to enhance sodium transport across the frog skin (Watlington, 1969) and potassium efflux from isolated fat cells (Perry and Hales, 1970). A close relationship between cAMP and intracellular calcium concentrations has also been postulated (Rasmussen and Tenenhouse, 1968).

The visual excitation process obviously needs a transmitter between photon capture on the disc membrane and the conductance change of the cell membrane. Cyclic AMP could be a possible candidate for this transmitter role, if two basic conditions would be fulfilled:

- The two enzymes which regulate the intracellular cAMP concentration, adenylate cyclase and phosphodiesterase, should be present in photoreceptors.
- The intracellular cAMP concentration must be regulated by light. Thus either adenylate cyclase or cAMP-phosphodiesterase should be light-dependent. Moreover, a relatively low light intensity causing the bleaching of at most a few percent of rhodopsin, should change the

enzyme activity rather strongly to account for the sensitivity of the system that permits the detection of the capture of a single photon per rod.

The first reports on adenylate cyclase activity in photoreceptors (Bitensky et al, 1971; Miller et al, 1971) mention an extremely high specific activity, which could be nearly completely inhibited by illumination. In view of the obvious importance of these findings for the theory of visual excitation, we decided to repeat and extend these experiments.

2.2. METHODS

2.2.1. Rod outer segment isolation

Frog rod outer segments are prepared in two different ways: by shaking off the outer segments in saline (Method I) and by flotation on sucrose (Method II: Bitensky et al, 1971). The first method is much faster and produces a preparation less contaminated by cellular debris. In both methods the frogs (Rana temporaria and Rana esculenta) are dark-adapted for at least 2 h. All subsequent operations, except for deliberate illumination, are performed under dim red light. After decapitation the retinas are extruded through a slit in the cornea and treated according to one of the following methods.

Method I: the retinas are collected in ice-cold modified Ringer solution, containing 112 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 3 mM ATP sodium salt and buffered to pH 7.4 with 10 mM Tris-HCl. They are then gently shaken for 1 min and filtered over a stainless steel wire screen (60 mesh). In some cases the suspension is centrifuged for 5 min at 4000 x g and the sediment suspended again in Ringer solution.

Method II: the retinas are collected in ice-cold 47.6% sucrose (0.1-0.2 ml per retina) and agitated vigorously during 2 min on a Vortex mechanical vibrator. The suspension is centrifuged at 100 000 x g for

90 min at 4°C in a Spinco SW 39 rotor. The viscous paste of rod outer segments on top of the solution is collected with a spatula.

The suspensions are freshly prepared before each assay and homogenized in various ways indicated under Results.

Bovine rod outer segments are prepared by means of sucrose density gradient centrifugation (de Grip et al, 1972). Bovine eyes (30-40) are placed immediately after death at room temperature in a light-tight container and are dissected within 2 h. The retinas are mildly homogenized in 15 ml ice-cold Tris-HCl buffer (0.16 M, pH 7.1) by means of a loosely fitting Potter-Elvehjem homogenizer. The homogenate is filtered through 120-mesh stainless steel wire screen under cautious stirring. The residue is washed with the same Tris-HCl buffer and the combined filtrate represents whole retinal homogenate. For isolation of outer segments this homogenate is mixed with 66.7% aqueous sucrose to a final concentration of 0.42 M and a final volume of 52 ml. With this suspension and an equal volume of 40% aqueous sucrose solution, two continuous gradients with a density range of 1.05-1.18 (0.42-1.38 M) are prepared. After centrifugation in a swing-out rotor (1 h, 27 000 x g, 10°C) a heavy sediment and two layers are obtained with densities of approximately 1.10 and 1.13. The upper layer at d $\sim$ 1.10 contains the rod outer segments. Both layers are collected and stored at -20°C. After thawing, the suspensions are routinely sonicated (3 times for 1 sec with a Branson B12 sonifier with microtip at maximal output).

2.2.2. Illumination

The outer segment preparation is illuminated either in ice during 10 min by a 100W tungsten lamp through 3 mm thick GG3 and OG2 filters (Scott-Jena) or by 1-3 flashes from a Rollei Strobofix E60 flash lamp through an OG2 filter. In the latter case the light intensity is regulated by varying the distance between lamp and suspension.

Before and after illumination 150- µl aliquots are taken to determine the rhodopsin content. The 150- µl aliquot is mixed with 125 µl

Ringer solution and 30 µl 10% Triton X-100. After centrifugation (10 min, 8000 x g) 200 µl supernatant is transferred to a cuvet with 1-cm light path and 10 µl hydroxylamine (1 M) is added. The 500-nm absorbance is determined before and after illumination during 5 min by a 100W tungsten lamp through GG3 and OG2 filters. Comparison of the 500-nm absorbance change ($\Delta A_{500\ nm}$) of a non-illuminated sample with the $\Delta A_{500\ nm}$ of the bleached samples yields the percentage bleaching at a given light intensity.

2.2.3. Enzyme assays

Adenylate cyclase activity is assayed in dim red light at 30°C with a reaction volume of 25 µl and a standard incubation time of 10 min. The medium contains 1.6 mM ATP, 5.3 mM aminophylline, 2.9 mM $MgSO_4$, 32 mM glycylglycine (pH 7.4) and an ATP regenerating system (2 mM phosphocreatine and 80 µg/tube creatinephosphokinase). The reaction is stopped by addition of 350 µl ice-cold 7% trichloroacetic acid. The cAMP formed during incubation is determined by means of saturation analysis (Brown et al, 1971), based upon the binding of cAMP to a cAMP-dependent protein kinase. Immediately after termination of the incubation 50 nCi ^{3}H-labelled cAMP is added. The sample is centrifuged and the protein washed with 5% trichloroacetic acid and water. The combined supernatants are chromatographed on Dowex (50 W x 8, 200 - 400 mesh) ion exchange resin columns (Cooke et al, 1972). The cAMP containing fractions are assayed using the cAMP-binding protein from bovine adrenals. Recovery of ^{3}H-cAMP is usually 70-80% and never falls below 50%. The formation of cAMP during incubation is linear with time for at least 20 min. The relative standard error for triplicate determinations is 8%.

(Na-K)-ATPase and Mg-ATPase activities are determined by means of a standard method used in our laboratory (Bonting, 1970). The tissue preparation is lyophilized and before assay the dry material is reconstituted with distilled water. Aliquots of 20 µl are mixed with 300 µl of either one of the media A and E and incubated for 15-60 min at 37°C. The activity in medium A (2 mM ATP, 55 mM NaCl, 5 mM KCl, 2 mM

$MgCl_2$, 100 mM Tris, 0.1 mM EDTA) represents total ATPase activity, while activity in medium E (2 mM ATP, 60 mM NaCl, 2 mM $MgCl_2$, 100 mM Tris, 0.1 mM EDTA and 0.1 mM ouabain) represents the Mg-ATPase (Na- and K-insensitive) activity. The difference between these two activities represents the (Na-K)-ATPase activity.

5'-Nucleotidase is determined according to Heppel and Hilmoe (1955). The incubation medium contains 1 M glycine-NaOH buffer (pH 8.5), 0.1 M $MgCl_2$, 2.4 mM 5'-AMP. After addition of enzyme, 150 µl of this medium is incubated for 15 min at 37°C. The reaction is terminated by addition of 50 µl 20% trichloroacetic acid. Inorganic phosphate is determined as in the ATPase assay (Bonting, 1970).

Alkaline phosphatase is assayed according to Wöltgens et al (1970) and succinate dehydrogenase according to King (1967) using phenazine methosulphate as an artificial electron acceptor.

Protein is measured by the method of Lowry et al (1951) using bovine serum albumin as a standard.

2.2.4. Materials

Adenosine 5'-triphosphate (ATP), adenosine 3',5'-monophosphate (3',5' cyclic AMP), creatinephosphate and creatinephosphokinase (rabbit muscle) are obtained from Boehringer, Mannheim, Germany, bovine serum albumin from Behringwerke AG, Marburg/Lahn, Germany, Dowex 50 W x 8, 200 - 400 mesh from Fluka AG, Germany and all-trans retinaldehyde from Eastman Kodak Company, Rochester, New York. Adenosine-8-^{3}H-3',5'-monophosphate, ammonium salt (24.1 Ci/mmol) is received from the Radiochemical Centre, Amersham, Nottingham, England.

All other reagents are of analytical grade.

2.3. RESULTS

2.3.1. Adenylate cyclase activity in rod outer segments

With suspensions prepared according to method I, the adenylate

cyclase activity in frog rod outer segments depends to a large extent upon the method of homogenization (Table I, column 2). While all

Table I. Adenylate cyclase activity in frog rod outer segments, prepared and treated in different ways. Method I: outer segments isolated by gentle shaking in Ringer, followed by filtration through wire screen and centrifugation. Method II: outer segments isolated by vigorous shaking, followed by sucrose flotation.

Homogenization method	Activity in nmole cAMP/mg protein per 10 min	
	Method I	Method II
Repeatedly passing through injection needle in water	0.067	0.041
Glass on glass in glycylglycine buffer	0.022	0.49 ± 0.085 (4)
Glass on glass in Tris buffer	0.015	-
Glass on glass in Tris buffer, followed by freezing and thawing	0.024	-
Sonication in Ringer	0.11	0.31
Sonication in 40% sucrose	-	0.52 ± 0.13 (7)
Range	0.015 - 0.11	0.041 - 0.52

activities are very much lower than those reported by Bitensky et al (1972a), the highest activity (0.11 nmole cAMP/mg protein per 10 min) is obtained after sonication. With suspensions prepared according to the method of Bitensky et al (1971, Method II), we obtain somewhat higher activities (Table I, column 3), which again depend on the method of homogenization. The highest activities are obtained by sonication in 40% sucrose and by glass on glass homogenization, 0.52 ± 0.13 and 0.49 ± 0.085 nmole cAMP/mg protein per 10 min, respectively, representing only 0.8% of the average activity for frog reported before (Bitensky et al, 1972a). There exists a considerable variation in activity between the different samples. For seven samples isolated by Method II and treated by

sonication in 40% sucrose the relative standard error is 25%, much larger than the 8% relative standard error for triplicate assays. In these suspensions a high sucrose concentration is necessary during the assay in order to obtain maximal activity. When the samples are diluted with water before assay, the activity decreases to 20% of that obtained by dilution with sucrose.

Most experiments on frog rods have been done with sonicated suspensions obtained by Method II, because this results in the highest activities and also because during the other homogenization procedure, which gives satisfactory activities (glass on glass homogenization), the inhibition by light is lost (see next section).

The adenylate cyclase activity has also been determined in cattle rod outer segments prepared according to a method developed in our own laboratory (de Grip et al, 1972). In this method the gradient centrifugation of a retina homogenate yields two layers in addition to a pellet of heavy material. The two layers each contain about 3% of the protein present in the pellet. The upper (purple) layer contains the rod outer segments (rhodopsin), the lower layer contains no rhodopsin. Table II shows the distribution of the adenylate cyclase activity over both layers and the whole retina, the specific activity in the outer segment layer being only 1/10 as high as in the lower layer and 1/4 as high as in the whole retina.

The lower layer is further characterized by determining the activities of five other enzymes in both layers and in whole retina (Table II). The rod outer segment layer is not contaminated with mitochondria as shown by the absence of the mitochondrial marker enzyme succinate dehydrogenase. The lower layer contains, in addition to mitochondria, plasma membrane fragments as indicated by the relatively high activities of the last four enzymes, which are all marker enzymes for the plasma membrane (De Pierre and Karnovsky, 1973). It is as yet not clear which retinal cell types contribute to this layer. However,

Table II. Enzyme activities determined in cattle retinas and two fractions obtained by gradient centrifugation

	Whole retina	Upper layer (rod outer segments)	Lower layer
Adenylate cyclase *	0.80	0.22 ± 0.05 (5)	2.30 ± 0.30 (4)
Succinate dehydrogenase **	4.90 ± 1.30 (2)	0 (4)	12.70 ± 2.10 (5)
(Na-K)-ATPase ***	2.50 ± 0.32 (4)	0.19 ± 0.02 (3)	5.10 ± 0.22 (4)
Mg-ATPase ***	1.0 ± 0.18 (5)	0.11 ± 0.01 (8)	2.30 ± 0.20 (4)
Alkaline phosphatase ***	0.50 ± 0.10 (4)	0.20 ± 0.02 (6)	1.15 ± 0.10 (6)
5'-Nucleotidase †	0.63 ± 0.12 (4)	0.30 ± 0.02 (5)	1.45 ± 0.20 (6)

* Expressed as nmoles cAMP/mg protein per 10 min

** Expressed in arbitrary units

*** Expressed in moles ATP/mg protein per 10 min

† Expressed in moles 5'-AMP/mg protein per 10 min

based on the tentative conclusion of Hagins (1972), that a large dark current of sodium 'is carried into the outer segments and extruded again by an ouabain-sensitive sodium pump, located somewhere along the inner segments and cell bodies', one could speculate that the very high (Na-K)-ATPase activity of the lower layer indicates the presence of a large amount of inner segment plasma membrane.

It is possible to fractionate the retinal homogenate, especially the upper layer, still further (Zimmerman, to be published). The retinas are homogenized in 0.3 M sucrose and centrifuged for 3 min at 800 x g. The supernatant is centrifuged 30 min at 17 000 x g and the pellet is resuspended in 0.3 M sucrose and layered on a linear sucrose gradient. After centrifugation at 75 000 x g for 13 hr, a rhodopsin profile is obtained as depicted in Fig. 10. Apparently the adenylate cyclase

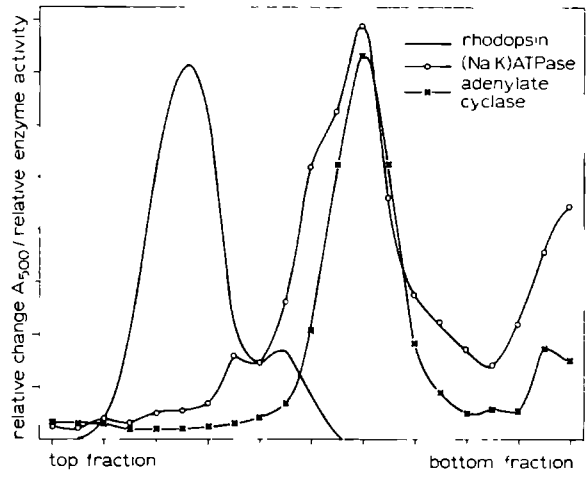


Fig. 10. Fractionation of a cattle retinal homogenate on a sucrose density gradient: distribution of adenylate cyclase and (Na-K)-ATPase compared to rhodopsin. After Zimmerman.

activity can be separated to a large extent from the rhodopsin (and thus presumably from the rod outer segments), while a somewhat larger part of the (Na-K)-ATPase activity remains in the rhodopsin containing fractions. Thus it seems possible to obtain rod outer segments almost completely free of adenylate cyclase activity.

2.3.2. Effect of illumination

The effect of illumination on the adenylate cyclase activity in rod outer segment suspensions of both frog and cattle (upper layer) is determined at different light intensities. All illumination experiments are performed with sonicated suspensions, since it appears that glass on glass homogenization destroys the light dependence, as reported before by Bitensky et al (1972a).

Both in frog and cattle rod outer segments there is a significant inactivation by light, especially at higher intensities. Since there is no basic difference between the light effects on the two species, the combined results of all illumination experiments, grouped according to

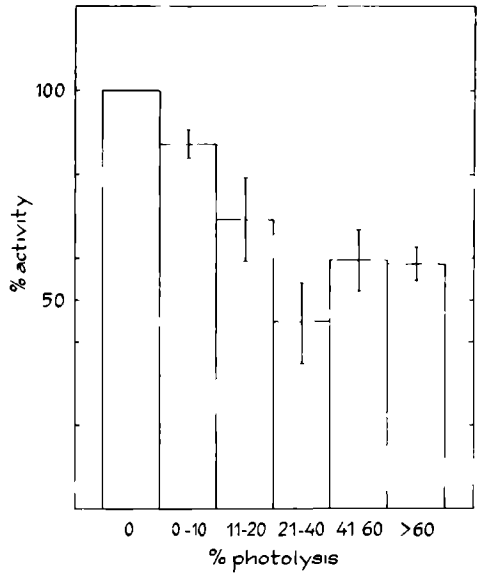


Fig. 11. Inhibitory effects of illumination on adenylate cyclase activity in rod outer segment suspensions. Combined results of all experiments (total 36) on frog and cattle.

the degree of bleaching of rhodopsin, are shown in Fig. 11. Complete inhibition is never observed. The average maximal decrease at extensive illumination is 50% of the original activity. Only one preparation out of twelve tested (included in the bar representing 21-40% bleaching) shows a larger inhibition (80%). At low bleaching levels (5% or less) we find no significant decrease in activity.

2.3.3. Further characteristics of cattle retina adenylate cyclase activity

The influence of calcium ions and EGTA on the adenylate cyclase activity in the rod outer segments (upper layer) obtained from cattle retina homogenates has been examined. Table III shows that addition of calcium ions to the assay medium inhibits the activity, while addition of 0.1 mM EGTA increases the activity. (EGTA, ethyleneglycol-bis-(α- aminoethyl-ether)-N,N'-tetraacetic acid, is a metal chelator, which shows a specificity towards calcium, as compared to other

Table III. Effects of calcium ions and EGTA on adenylate cyclase activity in rod outer segments (upper layer) isolated from cattle retina. Activities expressed in percent of the activity in a medium containing 1.6 mM ATP, 5.3 mM aminophylline, 2.9 mM $MgSO_4$, 32 mM glycylglycine, pH 7.4, 2 mM phosphocreatine and 80 µg creatine phosphokinase.

Ca^{2+} added (M)	- EGTA (%)	+ EGTA (10^{-4}M) (%)
0	100	158
10^{-6}	97	162
10^{-5}	93	158
10^{-4}	88	129
10^{-3}	28	24
10^{-2}	-	4

biologically important divalent cations (Marhol and Cheng, 1970). The latter increase can be suppressed by addition of calcium ions. When the concentration of calcium exceeds the EGTA concentration, stimulation changes into inhibition.

Addition of EGTA to the second layer also increases the adenylate cyclase activity. Addition of 10 mM NaF, which normally stimulates adenylate cyclase activity in other tissues, inhibits the activity in the rod outer segment layer by 35%. This is a very uncommon phenomenon, since it normally stimulates the adenylate cyclase activity as it does here in the lower layer (by 40%).

Table IV shows that illumination has no effect on adenylate cyclase activity of the lower layer, but that a 3:1 mixture (on protein basis) of upper and lower layer shows a significantly larger decrease in activity than expected if only the upper layer activity were inhibited by light. This decrease (35.0 - 24.2 = 10.8) is even larger than the entire upper layer dark activity present (8.3). This means that in the presence of upper layer material the activity of the lower layer is also affected by light. It suggests that a diffusible factor, released from

Table IV. Effect of light on adenylate cyclase activity in upper and lower layers from cattle retina. Results in pmoles cAMP/10 min per tube, averages of three determinations, each with standard errors

	Upper layer	Lower layer	Mixture		P-value difference calc'd-obs'd
			Calculated	Observed	
Dark	8.3 ± 0.4	24.3 ± 1.2	32.6 ± 1.3	35.0 ± 2.9	0.5
Illuminated*	5.1 ± 0.7	25.3 ± 0.9	30.4 ± 1.1	24.2 ± 0.1	0.001

* Causing 50% bleaching of rhodopsin

the upper layer material, causes the inhibition of the adenylate cyclase activity by light.

The possibility that the diffusible factor might consist of calcium ions has been tested by addition of EGTA (0.05, 0.1 or 0.5 mM) before illumination. The light inhibition is not decreased in this case. Another likely possibility is that the diffusible factor might consist of all-trans retinaldehyde released from rhodopsin upon illumination. However, addition of 5 moles all-trans retinaldehyde (15 mM in methanol) per mole rhodopsin, followed by assay in darkness, gives the same adenylate cyclase activity in upper or lower layer as obtained upon addition of the solvent (0.5% final concentration) alone. Neither does addition of NADPH (5 moles per mole rhodopsin), which rapidly reduces free retinaldehyde to retinol, affect the inhibition by light. These findings plead against release of calcium or retinaldehyde as an explanation for the light inhibition of the adenylate cyclase activity.

2.4. DISCUSSION

At the time we started our investigation, only one short report had been published (Bitensky et al, 1971), which claimed the presence in rod outer segments of an adenylate cyclase with extremely high specific activity. Our aim has first been to check this report and secondly to

establish a possible role for this enzyme in visual excitation.

The adenylate cyclase activities have been measured by means of a binding assay, i.e. the amount of cAMP formed is determined by its binding properties to a cAMP-dependent protein kinase isolated from bovine adrenal gland. This method appears to give reliable results in our hands. Internal cAMP standards are quantitatively recovered (93 - 104%) and we find the same adenylate cyclase activity in rat pancreas (0.045 nmol cAMP/mg protein per 10 min) as obtained by Rutten et al (1972). Moreover, for whole cattle retina we obtain an activity of 0.8 nmol cAMP/mg protein per 10 min against 0.33 for whole calf retina reported by Brown and Makman (1972). In both reports the adenylate cyclase activity is measured as the conversion of ^{32}P-ATP into ^{32}P-cAMP, which is then separated by means of thin layer chromatography.

It is therefore surprising that the maximal activities observed by us (1.1 and 0.4 nmol cAMP/mg protein per 10 min in frog and cattle, respectively) are only a small fraction of the activities originally claimed by Bitensky et al (1972a), which range from 33 to 78 nmol cAMP/mg protein per 10 min for cattle and frog (Rana catesbeiana) respectively. In an attempt to resolve this discrepancy we have used various methods for isolation and homogenization of the rod outer segments, since it is known that adenylate cyclase activity is generally rather sensitive to the conditions of tissue preparation. While the adenylate cyclase activity is clearly dependent on the preparative methods used (Table II), the highest activity found by us is still only about 1% of those reported by Bitensky et al (1972a). Nevertheless, our values seem to be correct in view of the fact that later reports from the same laboratory show a 100-fold lower specific activity of the adenylate cyclase present in the rod outer segment preparations (Bitensky et al, 1973b; Miki et al, 1973, 1974). Hence, the later results of Bitensky et al for the specific activity are in agreement with our results. The reason for their earlier erroneously high results has not been given.

Table V. Adenylate cyclase activity reported in rod outer segments of different species. Activities expressed as nmole cAMP/mg protein per 10 min.

	frog	mouse	cattle	human
Pannbacker (1973)			0.30-2.20	
Chader et al (1973)			0.15	
Hendriks et al (1973)	0.52		0.22	
Miki et al (1973)	0.17			
Pannbacker (1974)				2.0
Lolley et al (1974)		0.35		
Manthorpe and McConnell (1974)			0.27	

More recently other reports have appeared showing the presence of adenylate cyclase activity in rod outer segment preparations of various species. Table V shows that the specific activities are all in the same range. From these data one could conclude that adenylate cyclase is indeed present in photoreceptors. However, the question arises whether this rather low activity might not be due to contamination with other retinal components containing a high activity of the enzyme. Our finding of a 10-fold higher activity in the lower layer from cattle retina (Table II) would seem to support this possibility. However, a second gradient centrifugation of the upper layer does not decrease the specific adenylate cyclase activity and NaF (10 mM) has opposite effects on the activities from both layers, which would suggest the contrary. The results, however, of a somewhat more refined fractionation of cattle rod outer segments (Fig. 10) show that it is possible to separate the adenylate cyclase activity nearly completely from rhodopsin (and thus the outer segment membranes), as has also been reported by Bownds et al (1974). Apparently, the purer the rod outer segment preparation is, the lower the adenylate cyclase activity. In fact, enrichment in rhodopsin content seems to be coupled to a decrease in adenylate cyclase activity

(Manthorpe and McConnell, 1974). It seems unlikely that rod outer segments lose endogeneous adenylate cyclase during isolation, since it is a firmly bound membrane enzyme and the relatively mild isolation conditions appear to make solubilization improbable.

Therefore, while a complete separation of adenylate cyclase and rod outer segment material has not yet been shown, it seems altogether probable that the adenylate cyclase in the rod outer segment fraction indeed derives from a contamination with other retinal material. This would also explain the large variation in the activity and the fact that in frog lower activities are found in method I, which in our opinion yields a purer preparation than method II.

In spite of this conclusion, the effect of illumination upon the adenylate cyclase activity has been examined. The first reports show that bleaching of nearly all rhodopsin inhibits the activity up to 90% (Miller et al, 1971), although bleaching of the first few percent of rhodopsin hardly decreases the activity. In later work (Miki et al, 1974) a complete inhibition is reported if only 2% of the rhodopsin is photolyzed. Our experiments (Fig. 11) show a maximal decrease in adenylate cyclase activity of 50% at maximal bleaching of rhodopsin, which is also reported by Chader et al (1973). It could be argued that the rhodopsin in the isolated outer segments has been partially bleached to start with. However, this cannot be the case for our frog preparations, since the animals have been dark adapted for several hours prior to sacrifice and the retinas have been treated in darkness or safe red light (> 610 nm). Furthermore, treatment of the cattle rod outer segments with 11-cis retinaldehyde during isolation to convert all opsin to rhodopsin (de Grip et al, 1972) does not lead to an increase in the dark activity as compared to a control, which is treated identically in the absence of 11-cis retinaldehyde.

This inhibitory effect of illumination alone does not prove the involvement of the adenylate cyclase in visual excitation. In order for the enzyme to play a primary role in this process, a large decrease in

activity at a minimal amount of bleaching seems required. However, at low bleaching levels (5% or less) we observe no significant decrease in activity. The results of the combination experiment (Table IV) suggest that it is more likely that the light effect is secondary and due to the release of an inhibitory factor by the photolysis of rhodopsin, e.g. calcium or all-trans retinaldehyde, although our experiments aimed at elucidating this matter do not confirm either possibility. The fact that the bleaching of material from the upper layer can lead to inhibition of the adenylate cyclase activity in the lower layer further shows that a light effect upon an enzyme activity is in itself no proof for the in vivo presence of the enzyme in the rod outer segments.

Another way to regulate the cAMP concentration would be the variation of the phosphodiesterase activity. A cAMP phosphodiesterase with a specific activity much higher than adenylate cyclase is reported to be present in different mammalian photoreceptors. This activity apparently can be stimulated by light if ATP is present in the incubation medium (Chader et al, 1973; Miki et al, 1973, 1974). This stimulation of phosphodiesterase activity would be the real cause for the observed inhibition of the adenylate cyclase activity. It appears, however, unlikely that the inhibition of adenylate cyclase encountered in our experiments is caused by this kind of stimulation. The optimal ATP concentration for this phenomenon is 0.1 mM, while at higher concentrations the effect of illumination is smaller. Our incubation medium contains 1.6 mM ATP, which concentration is kept constant during the assay by means of an ATP regenerating system. Moreover, the presence of a phosphodiesterase inhibitor in rather high concentration (5.3 mM aminophylline) should also minimize the effect of a change in phosphodiesterase activity. Finally, it should be mentioned that the fractionation of cattle retinas, described in the results section, leaves the cAMP phosphodiesterase activity in the same fractions as the adenylate cyclase activity, which is largely separated from the rod outer segments

(Zimmerman, to be published).

Taking all evidence together, we feel that it is unlikely that cAMP plays a direct role in visual excitation. It appears even highly questionable whether the adenylate cyclase activity occurs in the rod outer segments at all. If it is present in low activity, it apparently must be involved in another phase of the photoreceptor function. A role in recovery of photoreceptor sensitivity after bleaching has been suggested (Bownds et al, 1973), but recent experiments show no evidence for such a function (Hood and Ebrey, 1974).

The fact that the presence of cAMP metabolism in rod outer segments seems rather doubtful at the moment, does not mean that cyclic nucleotides are not involved in photoreceptor function at all. Another naturally occurring cyclic nucleotide is cGMP. Information regarding the biological importance of cGMP is still rather limited compared to our present knowledge of the role of cAMP. However, recently cGMP levels have been shown to be increased by biologically active agents and it appears that elevated cGMP levels are coupled to decreased cAMP concentrations (Goldberg et al, 1973). Both the cGMP producing enzyme, guanylate cyclase, and the cGMP hydrolizing enzyme are present in rod outer segment suspensions. The guanylate cyclase activity is higher than the adenylate cyclase activity, while the cyclic nucleotide phosphodiesterase shows a clear preference for cGMP as a substrate compared to cAMP. Moreover, both guanylate cyclase and cGMP phosphodiesterase show up in the same fractions as rhodopsin when retinal homogenates from cattle are fractionated on a sucrose density gradient (Zimmerman, to be published). Thus it appears that cGMP, rather than cAMP, may be involved in photoreceptor function.

CHAPTER 3

CALCIUM IN ISOLATED ROD OUTER SEGMENTS: CHANGES IN DISTRIBUTION UPON ILLUMINATION

3.1. INTRODUCTION

Calcium ions appear to play an important role in many physiological processes. There are good reasons for believing that calcium acts as a key regulatory ion and controls a wide variety of events at the intracellular and membrane level. This implies that generally the intracellular calcium ion concentration must be carefully controlled at a low level. In animal cells the concentration of calcium in the cytoplasm is normally in the range of 10^{-6} to 10^{-7}M (Baker, 1972), while the concentration in the extracellular fluid is close to 10^{-3}M. In order to maintain this gradient, the plasma membrane must possess mechanisms for extruding calcium ions actively from the cell, while the mitochondria and possibly the endoplasmic reticulum (and in skeletal muscle the sarcoplasmic reticulum) exert powerful intracellular buffering effects. In this way increases in intracellular calcium concentrations can trigger a particular hormonal, secretory or contractile response.

A regulatory role for calcium has indeed been proposed in a number of physiological functions:

- The most important and widely accepted roleof calcium is possibly that of serving as the coupling factor between excitation and contraction in all forms of muscle (Ebashi and Endo, 1968; Tonomura, 1973).
- Calcium is also essential to normal nervous function (Baker, 1972), where it stabilizes the excitable membrane (Koketsu, 1969) and couples excitation to secretion at nerve terminals (Hubbard, 1970; Rubin, 1970).
- It plays a dominant role as a stimulus-secretion coupling agent in a

great variety of tissues. The function of calcium in the secretion of macromolecules has been closely investigated in the catecholamine secretion from the adrenal chromaffin cell (Douglas, 1968) and in pancreatic enzyme secretion (Case, 1973).

- It has recently been postulated that calcium is involved in the regulatory action of cyclic AMP (Rasmussen, 1970) and cyclic GMP (Schultz et al, 1973), and therefore in hormonal action.

In addition to being transported across the membrane, calcium is a key component of the membrane itself (Koketsu, 1969). Changes in the amount of calcium bound to the membrane influence many of its physical properties, e.g. its permeability to water, other ions and solutes. A model of the cell membrane has, therefore, been constructed which assumes that the membrane exists in one of two stable states: a resting or calcium-associated state and an active or calcium-dissociated state. Excitation of the membrane induces dissociation of the calcium, which increases its permeability to sodium, potassium, calcium or other cations (Koketsu, 1969).

Recently the involvement of calcium in photoreception has been proposed. The model of Hagins (1972; chapter 1, section 1.2.4) for controlling the photocurrent in rod and cone outer segments also attributes a transmitter function to calcium, as it is known to possess in other tissues. So far, the evidence supporting this hypothesis is indirect and comes from electrophysiological measurements in the intact retina, involving variations in the medium calcium concentration (Yoshikami and Hagins, 1973; Hagins and Yoshikami, 1974). No physiological or biochemical evidence has been presented. Even hardly anything is known about the calcium content of rod outer segments and its intracellular distribution.

Therefore we have determined the calcium content of intact isolated frog and cattle rod outer segments. In order to obtain some idea about the intracellular calcium distribution, we have ruptured the

membranes of the outer segments by means of lysis and sonication, and measured the calcium distribution over particulate and soluble fractions. Our final intention has been to show an effect of light upon the calcium distribution in the outer segments.

3.2. METHODS

3.2.1. Rod outer segment isolation and treatment

Frog rod outer segments are isolated from the excised retinas of dark adapted frogs (Rana esculenta) by gentle shaking for 30 sec in a modified Ringer solution, containing 112 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 10 mM glucose, 3 mM ATP-disodium salt, and buffered to pH 7.4 with 10 mM Tris-HCl. After filtration over a stainless steel wire screen (60 mesh) the resulting suspension is centrifuged for 10 min at 1200 x g, the sediment is resuspended in Ringer solution and centrifuged again. The final sediment is suspended in the desired medium. The entire procedure takes less than 60 min.

Cattle rod outer segments are prepared as described in chapter 2 (section 2.2.1). After sucrose density gradient centrifugation the rod outer segment containing fractions are collected and diluted with one volume 0.16 M Tris-HCl, pH 7.1. After centrifugation (10 min at 4600 x g) the pellet is washed once more with the same Tris buffer.

Rod outer segments are broken into fragments by means of osmotic lysis or by sonication. Lysis is induced by addition of 4 vol H_2O (or 10 mM EDTA) to 1 vol rod outer segments suspended in isotonic medium, followed by vigorous shaking on a Vortex mixer for 10 sec. The membrane fragments are separated from the soluble material by centrifugation at 2900 x g (10 min). Sonication is carried out with a Branson B12 sonifier equipped with microtip by means of five 1 sec bursts at maximal output, with the preparation cooled in ice. Here separation of particulate and soluble material is accomplished by centrifugation at 9500 x g (10 min). Light microscopy indicates that both procedures cause complete

destruction of the typical outer segment structure. Only small fragments (maximal dimension about 5 μ) remain visible.

Illumination and determination of rhodopsin content in outer segment preparations are performed as described in chapter 2 (section 2.2.2).

The ionophore A23187 is a gift from Eli Lilly and Company. It is dissolved in acetone (29 mg/ml) and then 9 vol ethanol are added. Appropriate amounts of this solution are added to the Ringer solution.

3.2.2. Atomic absorption spectroscopy

Calcium and magnesium are determined with a Pye Unicam SP1950 double-beam atomic absorbance spectrophotometer. Supernatants are diluted with $LaCl_3$ to overcome possible anionic interference. The final concentration of $LaCl_3$, which is obtained as a solution for atomic absorption spectroscopy from BDH Chemicals Ltd. (Poole, England) is 0.5%. The solutions are used as such. Sediments are first dried at 110°C and digested for 2-3 h at 180°C in a mixture of H_2SO_4-$HClO_4$-HNO_3 (1:3:12, by volume) and then measured quantitatively in 0.5% $LaCl_3$ solution against appropriate standard solutions. The calcium standard solutions are made up from water-free $CaCO_3$ (E. Merck, Darmstadt, Germany), magnesium standards from $MgSO_4$ (ibid.). The experiments are performed in Hysil glass tubes, which are routinely washed before use with successively HNO_3 (at 100°C), double distilled H_2O, an EDTA solution and again double distilled H_2O.

3.3. RESULTS

3.3.1. Calcium content of isolated frog rod outer segments

Our isolation method reproducibly yields a suspension containing mainly intact rod outer segments, as examined by phase-contrast microscopy (Fig. 12). The suspension contains some mitochondrial contamination, because a minor part of the rods inevitably contains the myoid body. In addition, some melanin granules derived from the pigment epithelium are present. Addition of ATP to the Ringer medium during

Fig. 12. Isolated frog rod outer segments suspended in ATP-containing Ringer solution, viewed through a phase contrast microscope.

isolation appears to give a higher yield of intact outer segments. The rhodopsin yield is 0.99 (S.E.: 0.06, 69 determinations) nmoles rhodopsin per retina, which is nearly half the total rhodopsin (2.29 $\pm$ 0.09 nmoles, 6 determinations) present in the retina of this species.

The rod outer segment suspensions isolated in this way contain an appreciable amount of calcium. Fig. 13 shows that after two centrifugations the calcium content of the sediment is fairly stable, each further washing and centrifugation step removing only 2% of the remaining calcium. Neither centrifugation at a higher g-force (2900 x g), nor addition of 10 mM EDTA to the Ringer solution affects this picture in a significant way. Therefore we assume that all calcium present in the particulate fraction after the second centrifugation does indeed originate from the rod outer segments.

The average calcium content of the rod outer segment preparations is then 12.4 (S.E.: 0.8, 38 determinations) moles calcium per mol rhodopsin. When ATP is omitted from the Ringer solution during isolation, the calcium content is significantly lower (Table VI).

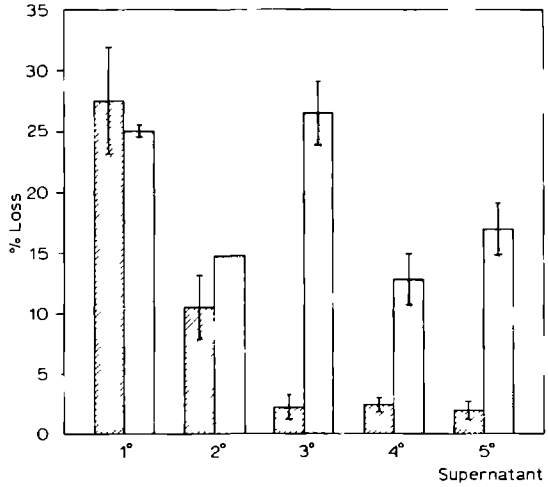


Fig. 13. Calcium loss from frog rod outer segment suspensions. Outer segments are shaken off in an ATP-containing Ringer solution, filtered and centrifuged at 1200 x g (10 min). The pellet is washed four times with the same solution (▨: n = 6). Alternatively, after the second centrifugation the pellet is resuspended and divided into four aliquots, which are centrifuged and washed two times with the same solution (□ : n = 4). Calcium is determined in the five supernatants and the pellet and the calcium loss is expressed as percent of the amount remaining in the sample after each centrifugation.

Table VI. Calcium content of frog rod outer segments isolated in various ways. The rod outer segment suspension is centrifuged after filtration, the sediment resuspended and centrifuged again. Calcium is determined in the resulting sediment and expressed as mol per mol rhodopsin with standard error of the mean. In parentheses the number of experiments (each in triplicate) is given. Statistical analysis by means of the Wilcoxon test gives the following P-values:
all values without sampling vs all values with sampling, $P < 0.001$
all values (+ATP) without EDTA vs all values with EDTA, $P = 0.38$
all values ($\pm$ EDTA) with vs all values without ATP, $P < 0.001$

Medium	without sampling	with sampling
Ringer, 3 mM ATP	12.4 $\pm$ 0.8 (38)	8.7 $\pm$ 1.5 (13)
Ringer, 3 mM ATP, 10 mM EDTA	12.8 $\pm$ 1.6 (4)	8.4 $\pm$ 1.6 (3)
Ringer, no ATP, no EDTA	8.0 $\pm$ 0.6 (11)	4.6 $\pm$ 0.8 (9)

Addition of 10 mM EDTA to the Ringer solution has no effect on the calcium concentration in the sediment, indicating that virtually all calcium is sequestered.

Table VI also shows that sampling of an outer segment suspension by means of a constriction pipette clearly produces a significant calcium loss from the outer segments, noticeable also in the higher calcium levels in the supernatant after centrifugation of the sample (Fig. 13). While after a normal isolation procedure (without pipetting) only 2% of the remaining calcium appears in the supernatant during a third washing and centrifugation step, this loss is increased to 27% after pipetting. Apparently pipetting damages a considerable fraction of the outer segments. This makes it difficult to obtain reproducible aliquots, as far as the absolute amounts of calcium in the sediments are concerned. We have, therefore, always determined calcium in both sediment and supernatant after centrifugation and have expressed the results (Tables VII-X) as the percentage of total calcium remaining in the sediment for each aliquot.

3.3.2. Effect of illumination on the total calcium content of frog rod outer segments

We have determined the effect of light on the total calcium content of the outer segments. Rod outer segments suspended in a Ringer solution are illuminated immediately after sampling, either by one or more flashes from a Rollei flash lamp (the first flash causing the bleaching of about 50% of the rhodopsin present) or by illumination with a 100W tungsten lamp for 5 min behind appropriate filters (yielding nearly 85% bleached rhodopsin).

Illumination by either method, with or without EDTA present in the Ringer solution, causes no significant change in the percentage of total calcium remaining in the sediment, as compared to nonilluminated controls (Table VII). Neither does illumination of outer segments suspended in an ATP-free Ringer solution, which would deprive a

Table VII. Effect of illumination upon the calcium content of intact frog rod outer segments. Isolated rod outer segments are washed twice with a Ringer solution and the sediment is resuspended and divided into 4-6 aliquots. Two or three aliquots are then illuminated, while the others are kept in darkness. After centrifugation calcium is determined in sediments and supernatants. Results are expressed as percent of total calcium remaining in the sediment.

Medium	Outer segment sediment		Ratio	No of experiments	Total no of determinations
	dark (%)	light (%)			
Ringer	68.3	72.4	1.06	10	50
Ringer + 10 mM EDTA	67.9	67.7	1.00	2	12
Ringer - ATP	56.1	57.9	1.03	5	30

possible calcium pump of its energy supply, result in a measurable change in calcium content. Therefore it appears that light does not change the overall calcium content of intact rod outer segments.

3.3.3. Calcium distribution in fragmented frog rod outer segments

In other cell types where calcium has a transmitter function (nerve, muscle), the calcium is largely sequestered in subcellular structures (mitochondria, sarcoplasmic reticulum). Therefore we have tried to establish the existence of separate calcium pools in outer segments by determining the calcium content before and after osmotic lysis or sonication. Calcium determinations after centrifugation show that calcium is indeed solubilized to a considerable extent after these procedures (Table VIII).

Lysis in 4 vol H_2O leaves only 45% of the original calcium content in the sediment, at least when the outer segments had first been suspended in an ATP containing Ringer solution. Suspending the outer segments in an ATP-free Ringer followed by lysis and centrifugation, leaves only 29% of the calcium in the sediment. When the outer segments

are lysed in 10 mM EDTA (or EGTA, which also chelates calcium, but much more specific than EDTA), almost all calcium is solubilized after centrifugation. Hence, the lysis method enables us to distinguish between a soluble and a particulate calcium fraction, the latter coinciding with the rhodopsin containing sediment. Since in the outer segments no other intracellular compartments than the rod discs are present, it is reasonable to assume that the particulate fraction represents calcium either present inside remaining intact discs or bound to disc membranes.

Table VIII. Calcium distribution in intact and fragmented frog rod outer segments. Isolated rod outer segments are washed twice with a Ringer solution, the sediment is resuspended and divided into different aliquots. After lysis or sonication the material is centrifuged and calcium is measured in sediment and supernatant. Results are expressed as percentage of total calcium in the sediment with standard errors of the mean.

Preparation medium	Ringer	Ringer + 10 mM EDTA	Ringer - ATP
intact outer segments	74.7 ± 4.7 (13)	68.6 ± 0.8 (3)	59.0 ± 5.7 (9)
after sonication (5 x 1 s)	59.5 ± 3.7 (6)	37.0 ± 2.4 (5)	36.2 ± 3.2 (2)
after lysis in 4 vol H_2O	45.8 ± 2.0 (27)	4.2 ± 2.2 (3)*	28.6 ± 3.4 (4)

* Lysis by addition of 4 vol 10 mM EDTA

Sonication also produces calcium loss from the particulate material. Omission of ATP from the Ringer solution enhances this effect. In contrast to the lysis procedure, however, addition of 10 mM EDTA during sonication does not solubilize all calcium, but still leaves about 40% of the calcium in the sediment after centrifugation, possibly indicating that not all membranes are ruptured or that a rearrangement to small vesicles has

occurred.

In view of the importance of the lysis procedure for the visualization of light dependent calcium movements in rod outer segments (see next section), attempts have been made to characterize this material somewhat further. It has already been mentioned that lysis in 4 vol 10 mM EDTA (or EGTA) solubilizes almost all calcium (Table VIII). Moreover, rod outer segments, lysed in H_2O and sedimented afterwards, lose all calcium upon treatment with a 10 mM EDTA containing Ringer solution. While intact outer segments lose only 5% of their calcium content during two consecutive washings and centrifugations in EDTA containing Ringer, lysed outer segments lose all their remaining calcium already during the first washing step (Fig. 14).

Another difference between intact and lysed outer segments is their behaviour upon treatment with the divalent cation ionophore A23187. While incubation of intact rod outer segments with this ionophore (final concentration 5 x 10^{-5}M) solubilizes nearly 50% of their calcium content

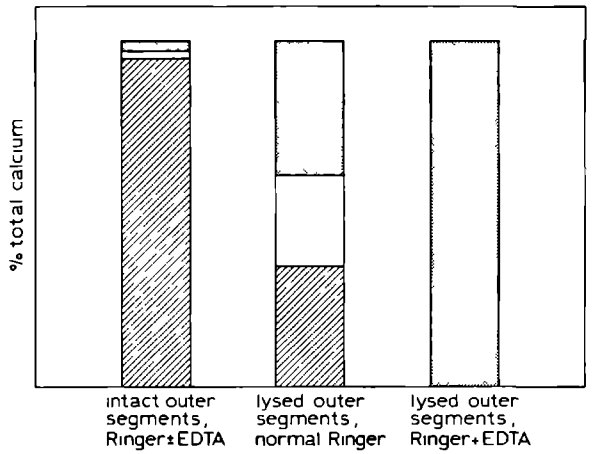


Fig. 14. Effect of EDTA-containing Ringer solution on the calcium content of frog rod outer segment suspensions. Intact or lysed outer segments are suspended in a Ringer solution and centrifuged at 2900 x g (10 min). The sediment is resuspended and centrifuged again. Calcium is measured in both supernatants and pellet and expressed as percent of total calcium: □, % in the 1th supernatant; □, % in the 2nd supernatant; ▨, % in pellet. The total bar represents 100%.

Table IX. Effect of ionophore A23187 on frog rod outer segment suspensions. Isolated rod outer segments are washed twice with an ATP containing Ringer solution, the sediment is resuspended and incubated in a Ringer solution, which contains 5×10^{-5}M A23187 (dissolved in ethanol-acetone, 9:1). Lysed rod outer segments are centrifuged first and then resuspended in the ionophore containing Ringer. After incubation (at room temperature) the samples are centrifuged and calcium is determined in supernatant and sediment. Results are expressed as percent of total calcium remaining in the sediment.

Incubation in	Intact outer segments	Lysed outer segments
Ringer + ethanol-acetone (9:1) solution, 30 min	90	90
Ringer + ionophore, 10 min	63	90
Ringer + ionophore, 30 min	52	91

(Table IX), the same ionophore concentration hardly affects the calcium level in the particulate fraction of a preparation of lysed outer segments (which contains the same amount of rhodopsin).

All lysis experiments described so far have been performed by the addition of 4 vol H_2O to 1 vol outer segments suspended in Ringer solution, decreasing the ionic strength suddenly by 80%. We have also investigated how a smaller decrease in ionic strength of the medium affects the calcium content of the rod outer segments (Fig. 15). It appears that decreasing the ionic strength by 20% does not affect the calcium content of the outer segment material at all. A decrease by 40% leads to a significant calcium loss, while lysis in 1.5 vol H_2O (60% decrease) has the same effect as lysis in 4 vol H_2O, about 40% of the calcium remaining in the sediment after centrifugation. The same amount of calcium remains bound to the particulate fraction, when the sediment is directly suspended in H_2O (not shown). Consequently the sedimented material has been washed once with a 10 mM EGTA containing Ringer solution. Here a gradual decrease of the amount of calcium remaining in the sediment can be seen with increasing lytic dilution (Fig. 15):

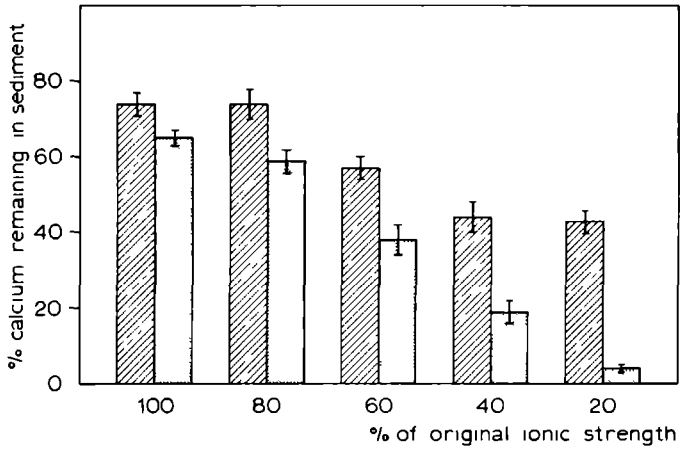


Fig. 15. Calcium loss from frog rod outer segments, induced by osmotic lysis. A suspension of intact outer segments in Ringer solution is diluted with the appropriate amount of H_2O, vigorously shaken for 10 sec on a Vortex mixer and centrifuged (10 min 2900 x g). The sediment is resuspended in an EGTA (10 mM) containing Ringer solution and centrifuged again. Calcium is measured in both supernatants and sediment. Results are expressed as percent of total calcium remaining in the sediment: ▨, after lysis; □, after washing the lysed material with EGTA-Ringer solution.

the more the ionic strength is decreased during the lysis procedure, the less calcium remains after treatment of the sediment with the EGTA containing Ringer solution.

3.3.4. Light-induced calcium movements in frog rod outer segments

We have determined the effect of illumination on the distribution of calcium between the soluble and particulate fraction both after lysis (mostly in 4 vol H_2O) and after sonication. When the intact rod outer segments are flash-illuminated and then lysed, a significant loss of calcium to the supernatant occurs, as compared to non-illuminated controls (Table X, upper row). This means that illumination causes a shift of calcium from the particulate to the soluble fraction in intact rod outer segments.

Having thus established that a particulate fraction of the outer

Table X. Loss of particulate calcium from frog rod outer segment preparations upon flash illumination. Isolated rod outer segments are washed twice with an ATP containing Ringer solution, the sediment is resuspended and divided into different aliquots. The aliquots are either lysed (with 4 vol H_2O) or sonicated (5 x 1 sec). Illumination is before lysis (first row), immediately after lysis (third row) or sonication (fourth row), or the suspensions are lysed or sonicated, resuspended in ATP containing Ringer after centrifugation and illuminated (respectively second and fifth row). After centrifugation calcium is determined in sediment and supernatant. Results are expressed both as percent of total calcium in the sediment and as the relative loss of calcium upon illumination.

Outer segment treatment	Dark sediment	Light sediment	% calcium lost upon illumination	No of exp.	Total no of determ.	P-value*
lysis after illumination	42.8	36.0	16	4	28	0.01
lysis followed by centrifugation and illumination in Ringer	38.9	28.7	26	5	31	< 0.001
lysis before illumination	41.5	39.0	6	3	22	0.85
sonication before illumination	60.3	61.6	-	3	17	0.47
sonication followed by centrifugation and illumination in Ringer	40.0	38.4	4	2	14	0.10

* Determined by applying two-way analysis of variance to the experimental data

segment suspension loses calcium upon illumination, the question arises whether the same fraction after destruction of the rod outer segments is still sensitive to light. Rod outer segments are lysed in darkness, the particulate material is sedimented and resuspended in isotonic solution.

Illumination again causes a significant release of the calcium remaining in the particulate material (Table X, second row). Thus the mechanism by which calcium is released by illumination remains intact upon lysis. However, when the rod outer segments are illuminated immediately after lysis, i.e. suspended in a 5-fold diluted Ringer solution, no significant effect of light is seen (Table X, third row). This suggests either that the low ion concentration (Na^+, K^+, Mg^{2+}) in the diluted Ringer solution or the relatively high calcium concentration in the medium after lysis (about 10^{-5}M) inhibits the light sensitive calcium release mechanism. Moreover, both magnesium and ATP appear to be important factors for this mechanism, since illumination of lysed outer segments in an ATP- or magnesium-free Ringer solution induces only half of the calcium loss that is obtained by illumination in a complete Ringer. When the outer segments are lysed in a milder way, by addition of 0.67 or 1.5 vol H_2O (cf. Fig. 15), no effect of light upon the calcium distribution between soluble and particulate fraction is seen.

Magnesium is also present in rod outer segments in rather high concentrations (1.4 moles magnesium per mol calcium). Upon lysis it also turns out to be divided into a particulate and a soluble fraction (particulate fraction: 18.1%, S.E.: 0,6, 4 determinations). Illumination of the lysed rod outer segments in a magnesium-free Ringer solution (required for accuracy of the magnesium determination) causes no change in the magnesium distribution (particulate fraction: 18.5%, S.E.: 0.6, 4 determinations), whereas 9.2% (S.E.: 0.2, 4 paired determinations) of the particulate calcium goes into solution. Although this calcium loss is only about half of the calcium loss in a magnesium containing Ringer solution, the effect is still significant. This finding indicates that the light-induced calcium release is specific for this bivalent cation.

In sonicated suspensions, no light-induced calcium movements can be found. For practical reasons, sonication after illumination is impossible, but illumination after sonication, either in the same Ringer

or in a fresh Ringer solution, does not induce a significant loss of particulate calcium (Table X, fourth and fifth row).

The light intensity applied in the foregoing illumination experiments causes photolysis of 85% of the rhodopsin. However, lower light levels still induce a loss of particulate calcium from lysed outer segment material (Table XI). Outer segments are lysed in 4 vol H_2O, centrifuged and the sediment is resuspended in an ATP containing Ringer solution and illuminated. The light intensity is varied by changing the distance between the sample and the flash apparatus. If the intensity is decreased so as to bleach only 15% of all rhodopsin, still the same amount of calcium is lost from the particulate material as when 85% of the rhodopsin is bleached.

Table XI. Loss of particulate calcium from lysed frog rod outer segments upon illumination with different light intensities. Isolated rod outer segments are washed twice with an ATP containing Ringer solution, the sediment is resuspended in the same Ringer and lysed with 4 vol H_2O. After centrifugation the sediment is resuspended in the Ringer solution and illuminated. Calcium is determined in supernatant and sediment. Results are expressed as the relative loss of calcium upon illumination with standard error of the mean.

% rhodopsin photolyzed	% particulate calcium lost
85	23.8 ± 1.2 (5)
40	19.4 (1)
15	26.9 ± 3.9 (5)

3.3.5. Calcium in isolated cattle rod outer segments

Some experiments have been performed with cattle rod outer segments, isolated by density gradient centrifugation. Isolation in isotonic Tris-HCl buffer yields outer segment suspensions which contain 2.5 (S.E.: 0.4, 11 determinations) moles calcium per mol rhodopsin. This suspension shows some of the characteristics of the frog outer segment suspensions. Both sampling and lysis in 4 vol H_2O cause a loss of

particulate calcium, leaving 76.6% (S.E.: 3.6, 5 determinations) and 44.5% (S.E.: 2.2, 3 determinations) of the total calcium, respectively, in the sediment after centrifugation. However, illumination causes neither a change in calcium content of intact outer segments nor a calcium loss from lysed outer segments, resuspended in Tris buffer. These results are not significantly altered when an ATP containing 'Ringer' solution is used instead of the Tris buffer.

3.4. DISCUSSION

3.4.1. Calcium content of isolated rod outer segments

It seems clear that rod outer segments contain a considerable amount of calcium, even after isolation. Frog outer segment preparations contain 12.4 moles calcium per mol rhodopsin, isolated cattle rod outer segments only 2.4 moles calcium per mol rhodopsin. While the calcium-rhodopsin ratio does not necessarily have to be the same in both outer segment species, this finding indicates that the time elapsing between death of the animal and completion of the isolation procedure, is an important factor in determining the final calcium content of the outer segment preparation. Frogs are killed in the laboratory (in the dark) and the retinas are immediately dissected and collected in an ice-cold isotonic solution. The final rod outer segment suspension used for the experiments, is produced within one hour from the death of the first animal. Cattle eyes, on the contrary, are obtained from the local slaughterhouse, where the eyes are stored for a few hours in a light-tight container at room tempercture. Isolation of outer segments by density gradient centrifugation also takes a few hours. Therefore, the purified outer segments are produced at least 5-6 hours from the time the first animal is killed. This probably means that a considerable part of the calcium may be lost from the cattle rod outer segments, as they also appear to lose 75% of their sodium and potassium content during isolation (Etingof et al, 1970). Therefore we

feel that the use of frog rod outer segments, although they appear to be somewhat less pure than cattle outer segments, approaches the in vivo situation more closely.

The possibility that during the relatively short isolation procedure, needed to obtain frog outer segments, still a part of the calcium ions is lost, can of course not be excluded. Yet, this can hardly be a significant part in view of the high final concentration in the rod outer segment suspension. Assuming that the rhodopsin concentration in the frog outer segment is 2.5 mM (Liebman, 1962), the calcium concentration must be about 31 mM. which is even higher than that in squid axon mitochondria (Baker, 1972). Our outer segment suspensions inevitably contain some mitochondria, as a minor part of the rods is seen to contain the myoid body (Fig. 12). However, it seems unlikely that the calcium content of these few mitochondria is responsible for the high calcium level in the rod outer segment suspensions. In addition, some melanin granules derived from the pigment epithelium are present. We have no quantitative information on the calcium content of the pigment epithelium. It is possible to decrease the amount of melanin granules in outer segment suspensions by including more filtration steps in the isolation procedure. However, this also dramatically decreases the yield of rhodopsin, thus making this procedure useless for routine determinations. When applied, the calcium-rhodopsin ratio in the outer segment suspension hardly changes during the filtration steps, while the contamination with melanin strongly decreases. This indicates that the pigment epithelium is not responsible for a significant part of the calcium present in the outer segment suspensions. The calcium in the final outer segment suspension cannot originate from the Ringer solution, which is essentially calcium-free, or from other retinal components. It is also unlikely that 'non-outer segment' calcium binds to the rod outer segment membranes during the isolation procedure, since washing the outer segment containing sediment with an EDTA containing Ringer solution does not decrease its calcium

level (Fig. 14). Thus it seems reasonable to assume that the rod outer segments indeed contain a large amount of calcium sequestered in their interior. The fact that isolation of the outer segments in ATP-free Ringer solution (Table VI) as well as fragmentation of the outer segments in ATP-free media (Table VIII) causes a significant decrease in the calcium level of the particulate material below that obtained in ATP containing media, suggests the presence of an ATP-driven calcium pump.

3.4.2. Effect of illumination on the total calcium content of frog rod outer segments

The calcium content of intact outer segments does not change upon illumination (Table VII). The absence of a measurable light effect does not rule out a light-induced release of calcium from the discs. It merely indicates that the permeability of the outer membrane to calcium is low, which is generally the case for cell membranes (Hurlbut, 1970; Schatzmann, 1970).

3.4.3. Calcium distribution in fragmented frog rod outer segments

Both sonication and osmotic lysis enable us to distinguish between a soluble and a particulate calcium fraction. In view of the absence of an effect of illumination on the calcium distribution in sonicated outer segments (Table X), these preparations have not been examined as extensively as the lysed outer segments.

Lysis of the outer segments, followed by centrifugation, leaves a considerable part of the calcium in the particulate fraction. This calcium therefore is either bound to the rod disc membrane or present within the disc. Lysis in the presence of EDTA or EGTA or with addition of EDTA or EGTA afterwards, removes all calcium from the outer segments, which finding might seem to favour binding to the membrane. This interpretation seems also consistent with the results of the experiments with the divalent cation ionophore A23187 (Reed and Lardy, 1972). This ionophore generally causes a release of calcium from within vesicular

fragments, but hardly affects the ATP-independent calcium binding (Entman et al, 1972; Scarpa et al, 1972). Therefore the fact that the ionophore has no significant effect upon the calcium content of lysed rod outer segments, while indeed decreasing the calcium content of intact rod outer segments (Table IX), may suggest that in the lysed outer segments the calcium is bound to the membrane fraction. However, the ongoing discussion on the mechanism of calcium release in stimulated nerve (Baker, 1972) and muscle (Ebashi and Endo, 1968) - release by downhill efflux vs release from calcium binding sites on membrane proteins - suggests that cautious interpretation is required.

3.4.4. Light-induced calcium movements in frog rod outer segments

In intact rod outer segments there appears to be an intracellular calcium movement upon illumination (Table X). This light-induced calcium release still appears after prior lysis followed by centrifugation and resuspension in isotonic medium, which procedure among other things removes the soluble calcium. It appears likely, in view of the discussion in section 3.4.3, that the calcium involved in the light effect is originally bound to the disc membrane.

The physiological significance of the light effect is indicated by the fact that bleaching of only 15% of the total rhodopsin content induces the release of the same amount of calcium as does bleaching of nearly all rhodopsin (Table XI). Assuming that 12.4 moles calcium are present per mol rhodopsin in intact rod outer segments (Table VI) and that 45.8% remains bound to the particulate material after lysis (Table VIII), it can be calculated from Table X (first row) that at nearly full (85%) bleaching 1.1 mol calcium per mol rhodopsin bleached, is solubilized. However, if only 15% of the rhodopsin is photolyzed, 6.1 moles calcium per mol rhodopsin bleached are lost from the particulate material and if the same effect should persist at bleaching percentages of about 1%, then a loss of about 100 moles calcium per mol rhodopsin photolyzed can be expected. Alternatively, one could speculate that the calcium which

is solubilized by the lysis procedure (46.2%), is present in or on the discs in vivo. If all this calcium would react like the particulate fraction obtained by lysis, then the amount of calcium lost upon illumination from the particulate material would be twice as large as calculated above (12 moles per mol rhodopsin bleached at 15% photolysis). The release of such amounts of calcium from the discs might indeed be able to close a sufficient number of the sodium channels in the rod outer membrane to trigger synaptic excitation. Yoshikami and Hagins (1973) estimate that the closing of at least 1% of the sodium channels is needed for this purpose.

Although we are completely convinced of the significance of our results, it should be mentioned here that not all experiments performed this way have been successfull, i.e. have shown light-induced calcium movements. All experiments described in this chapter have been carried out with a single batch of Rana esculenta. With a new batch of the same animal we have been unable to find the light effect. A similar experience has been mentioned by Szuts and Cone (1974, personal communication). Experiments with another frog species, Rana temporaria, also fail to show this light effect, although the outer segment suspensions show a similar calcium content and calcium distribution upon lysis. Possibly rather stringent, as yet undefined, conditions for the isolation of the outer segments and/or for the storage and feeding of the frogs, must be fulfilled before a preparation can be obtained, which functions optimally in this respect. Therefore, a careful investigation of these conditions is necessary before the light-induced calcium movements can be further characterized.

CHAPTER 4

CALCIUM BINDING BY CATTLE ROD OUTER SEGMENT MEMBRANES

4.1. INTRODUCTION

Assuming that photolysis of rhodopsin does indeed release calcium from the discs, the question arises whether this calcium originates from within the discs or from the disc membrane. In other words: is in the dark all calcium stored within the intradiscal space or is it (partly) bound to the cytoplasmic side of the disc membrane. Storage within the intradiscal space or on the inside of the disc membrane would imply that the membrane is equipped with a calcium transporting mechanism. Very active ATP dependent calcium transport systems have been found and characterized in membranes of sarcoplasmic reticulum (Weber et al, 1966; Inesi, 1972) and erythrocytes (Schatzmann and Rossi, 1971; Cha et al, 1971). Present concepts on the mechanism of active transport require that an essential step for calcium translocation is the passive binding of the ion to the membrane. Moreover, our results described in chapter 3 seem to indicate that at least part of the calcium released upon illumination, is bound to the disc membrane: apparently there exist light-sensitive calcium binding sites on rod outer segment disc membranes.

Therefore we have examined passive (ATP independent) binding of calcium to rod outer segment membranes. Calcium binding to rod disc membranes has hardly been investigated. Bownds et al (1971) mention an ATP stimulated calcium accumulation in suspensions of frog rod outer segments and Neufeld et al (1972) describe calcium binding by cattle rod disc membranes, which is doubled by ATP. No effect of light has been reported. We have investigated the calcium binding capacity of isolated cattle outer segment membranes by means of an equilibrium dialysis technique. Our principal aim has been to find out, whether light can in any way affect the calcium binding by disc membranes.

Calcium transport in sarcoplasmic reticulum and erythrocytes is probably mediated by an enzyme, (Mg+Ca)-activated ATPase (Hasselbach, 1974; Schatzmann and Rossi, 1971). The last part of this chapter describes some experiments aimed at detecting the presence of such an enzyme activity in rod outer segments.

4.2. METHODS

4.2.1. Isolation of cattle rod outer segments

Cattle rod outer segments are prepared by means of sucrose density gradient centrifugation as described in chapter 2. The upper layer from the gradient is diluted with one volume 0.16 M Tris-HCl, pH 7.1 and centrifuged (10 min at 4600 x g). The further procedure depends on the experiment which follows.

All equilibrium dialysis experiments are performed with fresh material, starting immediately after isolation. The preparations are washed twice with 15 mM EDTA and twice with double distilled water. The sediment is then taken up in the desired medium. In case a phosphorylation step is to be introduced before equilibrium dialysis, 1 mM EGTA is added to the above Tris buffer. The outer segments obtained after gradient centrifugation are washed once again with the EGTA containing Tris buffer; the sediment is resuspended, frozen and thawed. After centrifugation the sediment is taken up in the phosphorylation medium.

The outer segment material used for the ATPase assay is (after gradient centrifugation) washed twice with 15 mM EDTA and twice with double distilled water. The material is lyophilized and stored at -20°C prior to the enzyme assay (section 4.2.4).

The rhodopsin content of the preparations is measured as described in chapter 2 (section 2.2.2).

4.2.2. Phosphorylation of rod outer segments

Phosphorylation is performed essentially according to KUhn et al (1973). Isolated rod outer segments are suspended in a medium containing 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP (disodium salt), 0.1 mM ouabain, 2% sucrose and buffered to pH 7.4 with 20 mM Tris-HCl. The reaction volume is 40 ml, the protein concentration varies between 0.40 and 0.45 mg/ml. The suspension is incubated for 60 min at 37°C, either in the dark or under room light, and then centrifuged (10 min at 4600 x g). The sediment is resuspended in a medium containing 100 mM NaCl in 20 mM Tris-HCl (pH 7.4) and used for equilibrium dialysis.

Control experiments have been performed to confirm the phosphorylation of rod outer segment membranes under these conditions. γ-^{32}P-ATP (1.26 mCi/mmol, Radiochemical Centre, Amersham, Nottingham, England) is added to the reaction medium (final concentration 16×10^{-6}M). After various time intervals samples are taken and the amount of radioactive phosphate bound to the particulate material is determined after the filtration procedure described by KUhn et al (1973). After washing the filters, their radioactivity is immediately counted in 10 ml Aquasol (New England Nuclear) in a liquid scintillation counter.

4.2.3. Equilibrium dialysis

Isolated rod outer segments are suspended in the desired medium, usually 100 mM NaCl in 20 mM Tris-HCl (pH 7.4). The protein concentration ranges from 0.4 to 0.8 mg/ml. Samples of 5 ml suspension are placed in dialysis bags, consisting of Visking tube boiled in a solution containing 2 mM $NaHCO_3$ and 0.2 mM EDTA and thoroughly washed with double distilled water. The bags are closed, so that an air bubble is always enclosed to allow mixing of the contents. The closed bags are placed in test tubes (1.8 x 8.3 cm) containing 10 ml of an identical buffer solution to which approximately 0.67 µCi ^{45}Ca ($^{45}CaCl_2$, 445 mCi/mmol, Radiochemical Centre, Amersham, Nottingham, England) is added. The stoppered tubes are attached to a disc rotating vertically

at 1 rev/min. After dialysis for 40 h at 4°C in the dark or under room light, three 500- µl samples are taken from both the suspension inside and the solution outside the dialysis bag. The radioactive samples are mixed with 10 ml Aquasol and counted in a liquid scintillation analyzer (Philips). The total amount of calcium bound to the membrane fraction is calculated as follows:

$$\%\ ^{45}Ca_{bound} = \frac{(cpm_i - cpm_o) \times 10}{cpm_i \times 10 + cpm_o \times 20} \times 100\% \qquad (4\text{-}1)$$

$$\text{recovery}\ \%\ ^{45}Ca = \frac{cpm_i \times 10 + cpm_o \times 20}{cpm_T} \times 100\% \qquad (4\text{-}2)$$

$$\text{nmoles}\ Ca_{bound} = \text{nmoles}\ Ca_T \times \frac{\text{recovery}\ \%\ ^{45}Ca}{100} \times \frac{\%\ ^{45}Ca_{bound}}{100} \qquad (4\text{-}3)$$

where cpm_i and cpm_o are the total number of counts in 500 µl samples, from the dialysis bag and from the outside solution, respectively. The total amount of cpm present per tube (cpm_T) is calculated from a sample from the ^{45}Ca containing solution, taken before the start of the experiment. In this way the percent recovery of ^{45}Ca can be calculated (4-2). The recovery of ^{45}Ca in our experiments is always more than 90% and in most experiments even more than 95%, but always less than 100%. This is probably due to absorption to the dialysis bag and the glass tube. The number of nmoles calcium bound can now be calculated using Ca_T, the total amount of calcium present. Ca_T is the sum of the calcium added to the dialysis solution (^{45}Ca plus ^{40}Ca) and the endogeneous calcium from the outer segment material, which is determined by means of atomic absorption spectroscopy (chapter 3, section 3.2.2).

4.2.4. ATPase assay

The lyophilized outer segment material is reconstituted with distilled water and the ATPase activity is assayed in a medium containing 100 mM Tris-HCl (pH 7.5), 2 mM ATP (sodium or Tris salt) and

magnesium and/or calcium as described in the text. Samples are incubated for 60 min at 37°C. The reaction is terminated by addition of trichloroacetic acid and phosphate is determined as described elsewhere (Bonting, 1970).

Protein is measured by the method of Lowry et al (1951) using bovine serum albumin as a standard.

4.3. RESULTS

4.3.1. Calcium binding to cattle rod outer segment membranes

Our isolation procedure yields rather pure rod outer segment membranes, free of mitochondrial contamination (chapter 2, Table II). The average rhodopsin yield is 10.5 (S.E.: 0.5, 23 determinations) nmoles rhodopsin per retina. Rhodopsin constitutes 56% (S.E.: 2, 21 determinations) of the total membrane protein present. This value is calculated assuming a molecular weight of 39,000 and a molar absorbance of 40,000 for rhodopsin (Daemen et al, 1972) and using the protein concentration as determined by the Lowry method. This percentage is considerably lower than the values of 80-90%, commonly reported for pure rod outer segment membranes (Daemen, 1973). The reason for this discrepancy is probably that part of the rhodopsin in our preparations is photolyzed and that the Lowry method may give erroneously high values for the protein concentration.

After isolation, which includes washing with 15 mM EDTA, there is 0.32 (S.E.: 0.06, 21 determinations) nmol endogeneous calcium present per mol rhodopsin.

In our binding assay, equilibrium is reached after approximately 40 h of dialysis at 4°C. During this period, at the protein concentrations used, the volume of the outer segment suspension inside the dialysis bag does not change. This has been determined by adding dextran blue, which has a molecular weight of about 2×10^6 and should not pass the dialysis membrane, to the external dialysis solution

(10 ml). The absorbance at 620 nm is measured before and after the dialysis experiment. There is no significant change in absorbance, indicating that the dextran blue concentration, and thus the volume, has not changed. Therefore we may apply the equations from section 4.2.3 to calculate the calcium binding capacity of the rod outer segment membranes. The amount of calcium bound is directly proportional to the protein concentration according to our measurements.

The use of equilibrium dialysis in measure binding of cations to macromolecules, may lead to false results due to Donnan effects. This is particularly the case in solutions which contain only low concentrations of electrolytes. We have investigated the contribution of the Donnan effect in our binding studies by measuring the 'binding' of ^{22}Na to rod outer segment membranes in a medium consisting only of 20 mM Tris-HCl (pH 7.4). It seems reasonable to expect that at the very low sodium concentration ($\pm 10^{-8}$M) existing in this case hardly any binding to the membranes will occur. Therefore any concentration difference between inner and outer solution may be attributed to the Donnan effect. It appears that indeed a small difference arises at a protein concentration of 0.88 mg/ml, the Donnan ratio $r = \frac{[Na]^+_o}{[Na]^+_i}$ being 0.993. It follows that for calcium the ratio $\frac{[Ca]^{2+}_o}{[Ca]^{2+}_i}$ should be $r^2 = 0.986$, if the concentration difference between both solutions were only caused by the Donnan effect. However, when we substitute ^{45}Ca for ^{22}Na in our experiment, the ratio is much lower, ranging from 0.77 to 0.59 for protein concentrations between 0.41 and 1.02 mg/ml. This proves that under these conditions calcium is really bound to the membranes and that a Donnan contribution is negligible here.

Table XII shows the calcium binding capacity of cattle rod outer segments in various media. If the dialysis medium contains only 20 mM Tris-HCl, then in the dark 8.24 nmoles calcium are bound per mg protein

Table XII. Effect of illumination and medium composition on calcium binding by cattle rod outer segment membranes. Isolated outer segments are suspended in the appropriate medium and dialysed for 40 h at 4°C against a ^{45}Ca containing buffer solution of the same composition as the suspending medium. The experiments in the fourth column are performed under room light. Calcium binding is expressed as nmoles of calcium bound per mg protein with the standard error of the mean.

Dialysis medium	Calcium concentration (M)	Dark	Light	P-value*
20 mM Tris-HCl	10^{-5}	8.24 ± 0.80 (2)	7.16 ± 0.68 (3)	0.80
+ 100 mM NaCl	10^{-5}	1.45 ± 0.17 (7)	1.05 ± 0.25 (5)	0.15
+ 100 mM KCl	10^{-5}	1.10		
+ 100 mM NaCl	10^{-3}	57.36 ± 0.14 (2)	48.99 ± 4.59 (3)	0.20
+ 100 mM NaCl + 5 mM $MgCl_2$	10^{-5}	0.64 ± 0.14 (4)	0.46 ± 0.19 (2)	0.53
+ 100 mM KCl + 5 mM $MgCl_2$	10^{-5}	0.57		
			combined	0.032

* Determined by means of the Wilcoxon test

(or 0.70 mol calcium per mol rhodopsin) at a calcium concentration of 10^{-5}M in the medium. Addition of 100 mM NaCl to the dialysis medium decreases the calcium binding capacity to 1.45 nmol calcium per mg protein (or 0.12 mol calcium per mol rhodopsin). About the same amount of calcium is bound, when the 100 mM NaCl is substituted by 100 mM KCl (or by a mixture of 50 mM NaCl and 50 mM KCl). Addition of 5 mM $MgCl_2$ further decreases the amount of calcium bound.

All experiments have been performed with freshly isolated outer segment material. However, storage of the material overnight in a medium containing 100 mM NaCl and 20 mM Tris-HCl (pH 7.4), at 4°C or at -70°C, does not change the calcium binding capacity of the preparation.

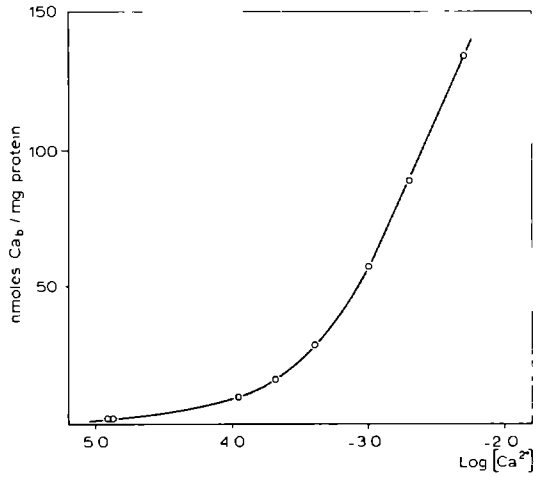


Fig. 16. Concentration dependence of calcium binding by cattle rod outer segment membranes. Isolated outer segments are suspended in 100 mM NaCl, 20 mM Tris-HCl (pH 7.4) and dialysed against the same ^{45}Ca containing buffer. The free calcium concentration is varied between 10^{-5} and 5×10^{-3}M. All experiments are performed in dim red light.

Lyophilization of the material has no significant effect either.

The results in Table XII (fourth row) indicate that an increase in the calcium concentration of the dialysis medium greatly enhances the calcium binding of the outer segment material. This is further shown in Fig. 16. Raising the calcium concentration from 10^{-5}M to 5×10^{-3}M, increases the calcium binding 130-fold. At 10^{-3}M calcium, already 57.4 nmoles calcium are bound per mg protein, which amounts to 3.5 moles calcium per mol rhodopsin. These calcium binding data are further analyzed by means of a so-called Scatchard plot. Scatchard (1949) has derived a method for plotting data on the binding of small molecules to macromolecules, which permits to determine by extrapolation the relative affinities and the number of binding sites. The plot is biphasic (Fig. 17), indicating the presence of two classes of binding sites. The extrapolated intercepts of the linear segments of the plot are used to calculate the number of binding sites and the association constant for

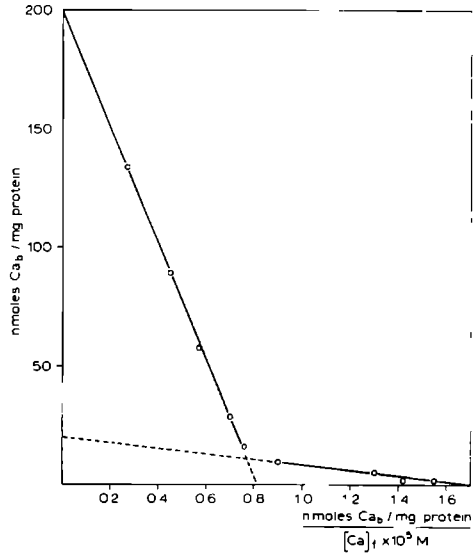


Fig. 17. Scatchard plot of calcium binding by rod outer segment membranes. The data from Fig.16 are plotted according to a method devised by Scatchard (1949).

each type of binding site. The intercept on the ordinate (n) represents the number of binding sites per mg of membrane protein. The intercept on the abcissa is nK where K is the association constant of the calcium binding site. The higher affinity binding sites for calcium, with an association constant of $1.2 \times 10^{4} M^{-1}$, accomodate 20 nmoles calcium per mg membrane protein. The lower affinity sites have an association constant of $2.4 \times 10^{3} M^{-1}$ and accomodate 200 nmoles calcium per mg protein. Assuming that 85% (cf. section 4.3.1) of the membrane protein is rhodopsin, this means that the maximal calcium binding capacity of rod outer segment membranes is approximately 10.5 moles calcium per mol rhodopsin.

Table XII also shows that illumination tends to decrease the calcium binding by 25% in each group of experiments. Although this decrease in each individual group is not significant, combined testing of the results with the Wilcoxon test (Bonting, 1952) shows an overall

significance (P = 0.032).

4.3.2. Effect of light-dependent phosphorylation on calcium binding by photorecptor membranes

Recently a light dependent phosphorylation of outer segment membranes has been reported (Kühn et al, 1973; Bownds et al, 1974). Since negatively charged phosphate groups might function as binding sites for calcium ions, we have examined calcium binding of cattle rod outer segment membranes after incubation under conditions, where phosphorylation might be expected to occur.

The protein kinase, which is necessary for phosphorylation, can easily be extracted from rod outer segment fragments (Kühn et al, 1973). Hence, the isolation method used for the experiments described in section 4.3.1, which involves washing with 15 mM EDTA, cannot be used. Instead we have used a milder isolation procedure (described under methods, section 4.2.1). The isolated rod outer segments are suspended in an ATP containing medium and incubated for 60 min at 37°C, either in the dark or under room light. After centrifugation the sediments are taken up in 100 mM NaCl - 20 mM Tris-HCl (pH 7.4) and submitted to equilibrium dialysis at 4°C in the dark. If incubation with ATP is performed in the dark, which presumably yields little phosphorylation, the outer segments bind afterwards 0.74 and 21.6 nmoles calcium per mg protein, respectively at calcium concentrations of 10^{-5}M and 10^{-3}M (Table XIII). This means that these outer segment suspensions bind less calcium than those, used in the earlier described experiments (cf. Table XII). However, direct comparison of these results is impossible, due to the different methods used for sample preparation.

If the samples have been illuminated during phosphorylation, a significant increase in calcium binding is seen after equilibrium dialysis, as compared to the non-illuminated controls. The average increase at a calcium concentration of 10^{-5}M is 0.40 nmol calcium per mg protein, which amounts to approximately 0.02 mol calcium per mol

Table XIII. Effect of phosphorylation on calcium binding properties of rod outer segment membranes. Isolated outer segments are incubated in a phosphorylating medium (see Methods) at 37°C for 1 h, either in the dark or under room light. After centrifugation the sediment is resuspended in 100 mM NaCl - 20 mM Tris-HCl (pH 7.4) and dialysed in the dark against the same ^{45}Ca containing buffer. Calcium binding is expressed as nmoles calcium bound per mg protein with the standard error of the mean.

Calcium concentration	Number of expts.	Dark	Light	P-value*
10^{-5}M	4	0.74 ± 0.03	1.14 ± 0.04	0.029
10^{-3}M	6	21.6 ± 3.3	39.9 ± 3.9	0.015

* Determined by means of the Wilcoxon test

rhodopsin. When the calcium concentration is raised to 10^{-3}M, the extra binding of 18.3 nmoles calcium per mg protein is equal to an increase of about 0.73 mol calcium per mol rhodopsin originally present. Isolated rod outer segments, which have been extensively washed with 15 mM EDTA and water, do not give an extra calcium binding after incubation in the light in a phosphorylation medium. This agrees with the assumption that extensive washing solubilizes the protein kinase necessary for phosphorylation.

We have investigated whether phosphorylation does indeed take place under our experimental conditions. γ-^{32}P-ATP is added to the medium and after various time intervals samples are taken and the degree of phosphorylation is determined after filtration and washing as described by Kühn et al (1973). In the light approximately 0.9 mol phosphate is incorporated per mol rhodopsin originally present, in the dark this figure is only 0.2. Since the resulting phosphate bound is rather stable in vitro (Kühn et al, 1973), it may be expected that during equilibrium

dialysis no phosphate groups are lost from the membranes. This would mean that in the outer segments which have been incubated in the light, an additional 0.7 mol phosphate per mol rhodopsin might be available for calcium binding, as compared to controls which have been incubated in the dark.

4.3.3. Ca-activated ATPase activity in rod outer segments

Sarcoplasmic reticulum and erythrocyte membranes contain an ATPase activity which apparently is involved in calcium transport (cf. Hasselbach, 1974). The relevant enzyme activity, correlating closely with calcium uptake, becomes apparent when calcium is added in low concentration to membranes suspended in a magnesium and ATP containing medium. We have studied the presence of such an enzyme activity in our cattle rod outer segment preparations.

It has already been shown in chapter 2 (Table II) that cattle rod outer segments, purified by density gradient centrifugation, contain

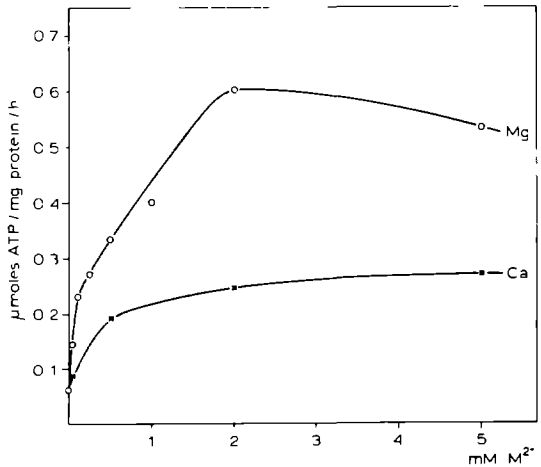


Fig. 18. ATPase activity in cattle rod outer segments. Lyophilized outer segments (isolated by density gradient centrifugation) are reconstituted with water and the divalent cation-activated ATPase activity is determined in a medium containing 2 mM Tris-ATP, 100 mM Tris-HCl (pH 7.5) and various concentrations of calcium or magnesium. Samples are incubated for 60 min at 37°C and the inorganic phosphate is measured as described by Bonting (1970).

Mg-ATPase activity. When lyophilized outer segments are suspended in 100 mM Tris-HCl (pH 7.5) and 2 mM Tris-ATP, an ATPase activity becomes apparent, which is dependent on the presence of magnesium (Fig. 18). This Mg-ATPase activity is maximal at a magnesium concentration of 2 mM and a magnesium-ATP ratio of 1. Calcium can substitute for magnesium (Fig. 18), but the Ca-activated ATPase activity in the presence of an optimal calcium concentration (5 mM), is only 45% of the activity obtained with 2 mM magnesium in the absence of calcium. Lineweaver-Burke plots, calculated from the values shown in Fig. 18 and corrected for the activity without divalent cation, do not yield completely straight lines. Thus it is impossible to calculate the exact K_m values. However, approximate K_m values are estimated to be 5×10^{-4}M and 4×10^{-5}M for magnesium and calcium respectively.

No evidence has been found for the presence of a (Mg+Ca)-activated ATPase. When calcium (0.01 - 10 mM) is added to the assay medium, which contains 2 mM magnesium and 2 mM ATP, stimulation of the ATPase activity is not observed. On the contrary, a gradual decrease in ATPase activity is observed with increasing calcium concentration (Fig. 19). When 5 mM

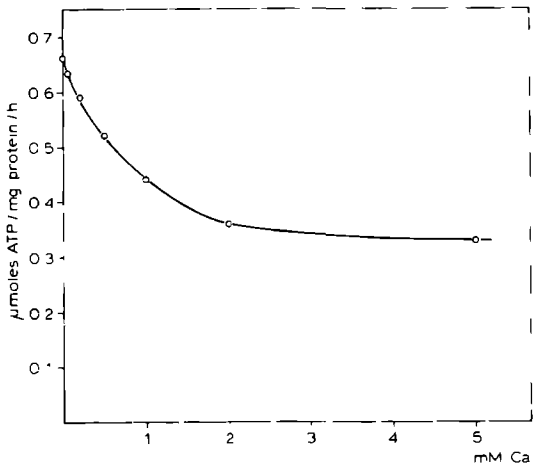


Fig. 19. Effect of calcium on the Mg-ATPase activity in rod outer segments. Calcium, in concentrations between 10^{-5} and 10^{-3}M, is added to a medium, containing 2 mM Tris-ATP, 2 mM $MgCl_2$ and 100 mM Tris-HCl(pH 7.5) and ATPase activity is measured as described in the legend of Fig. 18.

calcium is added to 2 mM magnesium, the ATPase activity approximately decreases to the level obtained when only 5 mM calcium is present. The same results are obtained, whether the incubation medium contains only 100 mM Tris-HCl and 2 mM ATP, or in addition 55 mM NaCl, 5 mM KCl, 0.1 mM EGTA and 0.1 mM ouabain. Hence, we find only a (Ca or Mg)-activated ATPase activity, but not a (Ca+Mg)-activated ATPase activity.

4.4. DISCUSSION

Isolated cattle rod outer segment membranes bind a fairly large amount of calcium. This binding appears to be passive, since no ATP or metabolic energy is needed.

The amount of calcium bound is strongly affected by the composition of the incubation medium. If only 20 mM Tris-HCl is present, a fairly large amount of calcium is bound, 8.2 nmoles per mg protein (Table XII). Addition of 100 mM NaCl to the Tris-HCl buffer decreases the calcium binding by more than 80%. The fact that even at a sodium-calcium ratio of about 10^4 a significant amount of calcium is bound, indicates the presence of rather specific calcium binding sites. Addition of 5 mM magnesium to the dialysis buffer decreases the calcium binding still further, at least at calcium concentrations of approximately 10^{-5}M (Table XII). At higher calcium concentrations (10^{-3}M) the effect of magnesium is much smaller (a decrease of 10%), again indicating the specificity of the binding sites for calcium.

There appears to exist an effect of light on calcium binding, when isolated outer segments are dialysed against a ^{45}Ca containing buffer solution. While the average values for binding in the dark (Table XII) are higher than for binding in light, the difference is only significant when a combined test of the results from the various media is made. An effect of light seems therefore certainly indicated, but more experiments are necessary to substantiate it. This result is in agreement with a very recent report (Hemminki, 1975), which shows a light-induced decrease in calcium binding by cattle rod outer segments.

It disagrees with results from Neufeld et al (1972), who find no effect of light upon calcium binding. However, their technique for measuring calcium binding, involving three centrifugation steps, is in our opinion less reliable than our equilibrium dialysis technique, where samples can be taken with minimal disturbance of the equilibrium state. The finding that illumination has only a relatively small effect upon the calcium binding capacity of isolated rod outer segments does not necessarily mean that a larger decrease cannot occur in vivo. It has already been pointed out that the isolation of cattle outer segments takes a rather long time and that outer segments may undergo structural and functional changes shortly after being separated from the retina (Robertson, 1966). This is further illustrated by the fact that a particulate fraction from frog rod outer segments, which can be isolated much faster, indeed shows a light-induced calcium loss under certain conditions (chapter 3).

The amount of calcium bound is strongly dependent on the calcium concentration in the dialysis medium (Fig. 16). Apparently two classes of binding sites exist (Fig. 17), high-affinity sites and low-affinity sites. Since calcium binding has not been studied below 10^{-5}M, the existence of a (small) number of sites with still higher affinity cannot be excluded. This result agrees fairly well with that reported by Hemminki (1975), at least as far as the number of binding sites is concerned. In our preparations the high-affinity sites can accomodate 20 and the low-affinity sites 200 nmoles calcium per mg protein, while Hemminki reports values of 25 and 210 nmoles calcium bound per mg protein, respectively. The association constants, however, differ considerably. Our outer segment membranes show a much higher affinity for calcium, since the association constant for the high-affinity sites is 1.2×10^4 M^{-1} in our preparations against 2.45×10^3 M^{-1} reported by Hemminki (1975), while the association constants for the low-affinity sites are 2.4×10^3 M^{-1} and 86 M^{-1}, respectively. These differences in affinity may quite possibly be caused by the difference in assay methods.

Presumably the sampling after our equilibrium dialysis method disturbs the equilibrium state to a lesser extent than the filtration method used by Hemminki. Taking samples by suction over Millipore filters might lead to a loss of bound calcium and thus to an apparently lower affinity than encountered in our experiments.

The presence of high-affinity sites seems typical for membranes involved in the active regulation of the intracellular calcium concentration, e.g. sarcoplasmic reticulum (Chevallier and Butow, 1971) and mitochondria (Reynafarje and Lehninger, 1969). Although the association constants of the high-affinity sites in these structures are higher than in our outer segment preparations, the presence of two types of sites and the large number of calcium binding sites suggests that rod disc membranes are involved in calcium binding and/or calcium uptake.

It seems likely that part of the calcium is bound to the negatively charged phosphate groups on the rod disc membranes. Since the number of phosphate groups is apparently regulated by light, illumination may be expected to regulate calcium binding via phosphorylation. Table XIII shows that the light-induced phosphorylation does indeed permit binding of an additional amount of calcium ions, in particular at higher calcium concentrations. In fact, when incubation in the light causes an extra binding of 0.7 mol phosphate per mol rhodopsin, subsequent equilibrium dialysis shows the binding of an additional 0.7 mol calcium per mol rhodopsin. This suggests that phosphorylation may play a role in dark adaptation by being part of a process which binds (or accumulates) calcium ions released upon illumination.

Thus phosphorylation of the outer segment membranes may be part of a calcium translocation mechanism. Calcium transporting membranes of sarcoplasmic reticulum (and erythrocytes) contain a (Mg+Ca)-activated ATPase activity. Their calcium translocation mechanism probably involves calcium binding after phosphorylation of the membrane by ATP (Weber, 1966). The ATPase activity which apparently is involved in calcium transport is the extra ATPase activity observed when small

amounts of calcium are added to a magnesium containing medium (Hasselbach and Makinose, 1961). Our investigation shows the presence in outer segment preparations of an ATPase activity which can function either with calcium or with magnesium, the optimal Ca-ATPase activity being only 45% of the optimal Mg-ATPase activity. Addition of calcium over a large concentration range (0.01 - 5 mM) to a medium which contains 2 mM magnesium, does not stimulate the ATPase activity, but results in a gradual decrease to the level which is obtained with 5 mM calcium alone (Fig. 19). Thus a (Mg+Ca)-activated ATPase activity, such as functions in sarcoplasmic reticulum, seems not to be present in rod outer segments. However, it is not altogether impossible that the ATPase activity evoked by the presence of calcium alone, has a function in the calcium binding by rod disc membranes.

In conclusion it can be stated that the experiments described in this chapter indicate that rod outer segment membranes contain a mechanism to bind a large amount of calcium. Illumination in the absence of ATP decreases the amount of calcium bound. In the presence of ATP light induces phosphorylation, which in turn enhances the calcium binding. Thus these mechanisms might form part of a cyclic process in which light induces a release of calcium and at the same time triggers phosphorylation to start the re-uptake of the calcium ions which have escaped from the discs.

An as yet unexplained phenomenon is the decrease in calcium binding in the experiments, where the effect of phosphorylation is studied, as compared to the first series of experiments. While in the experiments described in Table XII, the membranes bind 57.4 nmoles calcium per mg protein at a calcium concentration of 10^{-3}M, the membranes in the experiments described in Table XIII bind only 21.6 nmoles per mg protein under the same conditions. This difference may be due to the different isolation methods, but it is as yet unclear which steps are responsible for this phenomenon. The amount of endogeneous calcium remaining in the preparation is about the same in both methods.

CHAPTER 5

MOVEMENT OF CALCIUM IONS THROUGH ARTIFICIAL LIPID MEMBRANES

5.1. INTRODUCTION

Transport of calcium through photoreceptor membranes may be an integral part of photoreceptor function. In order to obtain more information about diffusion of calcium through biomembranes, we have studied calcium movements through model membranes. As phospholipid bilayers are generally considered to play an important part in the structure and function of biological membranes (Danielli and Davson, 1935; Hendler, 1971), presumably functioning primarily as a diffusion barrier and as a matrix for the membrane proteins (Singer, 1971), we have examined the calcium permeability of the basic phospholipid bilayer structure of biomembranes.

A suitable model system is formed by 'liposomes'. This model system derives from the fact that dry phospholipids of biological origins are liquid crystals, which undergo a sequence of spontaneous molecular re-arrangements in the presence of an aqueous solution of electrolyte or other solute, as the volume ratio of aqueous phase to phospholipid increases. These re-arrangements yield structures consisting of a series of concentric bimolecular membranes, each separated by an aqueous compartment. Since these are equilibrium structures it is thermodynamically probable that each and every bimolecular membrane forms an unbroken sheet, so that there is no exposed hydrocarbon-water interface. Therefore every aqueous compartment would be discrete and isolated from its neighbour by a closed membrane and the outermost aqueous compartment of the whole structure would be isolated from the continuous bulk aqueous phase. The solutes originally present in the aqueous solution are sequestered and can only diffuse and exchange between compartments and with the bulk aqueous phase by crossing the phospholipid bimolecular membranes (Bangham, 1968).

In general, the permeability properties of these liposomes demonstrate a great deal of similarity to those of the natural membranes. It has been shown that the permeation of cations and anions is remarkably similar for the model structures and the biological membrane (Bangham et al, 1965a). Moreover, the effects of narcotics (Chapman et al, 1967), local anesthetics (Papahadjopoulos, 1970) and the changes in ion permeability upon exposure of liposomes to lytic or protective steroids (Bangham et al, 1965b) are qualitatively the same as for natural interfaces. Also, the graded permeability of a number of non-electrolytes, as demonstrated by osmotic experiments, resembles that found for erythrocytes (Bangham et al, 1967). These close correlations support the concept that the barrier properties of liposomes have much in common with those of a biological membrane and that studies on these model systems can furnish useful information about the factors controlling the passive permeability to electrolytes.

Since calcium transport through biomembranes is generally thought to involve specific protein or peptide channels and possibly carriers, we have also introduced in our experiments calcium ionophores into the liposomes in an attempt to approach more closely the situation in a calcium transporting biomembrane.

This chapter describes experiments with liposomes prepared from retinal lipids, phosphatidylcholine and phosphatidylcholine plus phosphatidylserine. These liposomes are loaded with ^{45}Ca and different parameters of the leakage rate, such as time, temperature, calcium concentration and the effects of ionophores, have been examined.

5.2. METHODS

5.2.1. Lipid preparation

Retinal lipids are extracted from whole cattle retina. Two methods have been used based on the lipid extracting power of a chloroform-methanol mixture (Folch et al, 1957). Both are equally effective in

extracting lipid material. In the first method the material is extracted by three consecutive treatments with a chloroform-methanol-water mixture (20:10:1.2 by volume). The extraction (1 mg solvent per 10 mg material) is performed by continuous shaking on a Griffin shaker for 30 min under N_2, followed by centrifugation (10 min at 5900 x g) and isolation of the chloroform layer. The second method also employs a chloroform-methanol-water mixture. The membrane material is first homogenized in chloroform-methanol-water (5:10:4 by volume). The suspension is then vigorously shaken for 5 min and chloroform is added, raising the chloroform-methanol ratio to 1:1. After shaking the mixture for 1 min, water is added to a final chloroform-methanol-water ratio of 10:10:9. This suspension is shaken again for 1 min, centrifuged for 10 min at 5900 x g and the chloroform layer is collected. This procedure is repeated twice. In both procedures the combined chloroform extracts are washed once with 0.2 volume of 0.1 M KCl (to remove the remaining protein). After evaporation of the chloroform the lipids are dissolved in benzene-ethanol (4:1) and stored at -20°C under N_2.

Phosphatidylcholine is isolated from egg yolk according to Pangborn (1951) and phosphatidylserine from brain (Sanders, 1967). Both phospholipids are stored under N_2 in benzene-ethanol (4:1) at -20°C.

The phosphorus content of the lipid extracts is determined using a modified Fiske-Subbarow method after H_2SO_4-$HClO_4$ digestion (Broekhuyse, 1968).

5.2.2. Liposome preparation

Liposomes are prepared in the following way. Organic solvent is removed from 40 mg of phospholipid in a rotating evaporator. To the resulting thin film is added 4 ml of a salt solution containing: NaCl, 135 mM; $CaCl_2$, 0.15 mM (except in experiments where the internal calcium concentration is varied); Tris-HCl (pH 7.4), 10 mM; 0.1 mCi ^{45}Ca ($^{45}CaCl_2$, 445 mCi/mmol, Radiochemical Centre, Amersham, Nottingham, England). Suspension is achieved by vigorous mechanical shaking of the

mixture under N_2 for 30 min. The suspension is then left for 2 h at room temperature or overnight at 4°C.

In order to remove radioactive ions which have not been trapped in the liposomes, a gel filtration method is used (Bangham et al, 1965a). Two ml of the lipid suspension is passed down a column (2 cm x 23 cm) consisting of Sephadex G-50 equilibrated in a buffer containing 135 mM NaCl and 10 mM Tris-HCl (pH 7.4). The first and last lipid containing fractions, which may contain the largest and the smallest liposomes respectively, are discarded in order to obtain a relatively uniform liposome population. The diluted suspension of liposomes thus obtained, free of untrapped ^{45}Ca, is used for the leakage experiments.

5.2.3. Leakage experiments

Samples of 1 ml of suspension are placed in small dialysis bags (Visking tube, boiled in a solution containing 2 mM $NaHCO_3$ and 0.2 mM EDTA and thoroughly washed with double-distilled water) and the closed bags are placed in test tubes (1.3 cm x 13 cm) containing 9.5 ml of 135 mM NaCl in 10 mM Tris-HCl (pH 7.4). The stoppered tubes are attached to a disk, which rotates vertically at 1 rev/min. Over a period of at least 2.5 h 100- µl samples for radioactive counting are taken every 20 min from the outer solution and at the end of the experiment the radioactivity remaining inside the dialysis bag is also measured.

All radioactive samples are dissolved in 10 ml Aquasol (New England Nuclear) and counted in a liquid scintillation analyser (Philips).

In experiments, where ionophore is used, the ionophore solution is added to the liposome suspension immediately before it is poured into the dialysis bag. The ionophores X537A, a gift from Hoffman-La Roche, and A23187, a gift from Eli Lilly and Company, are dissolved in ethanol. Care is taken that no more than 5 µl ethanol is added per ml lipid suspension, since higher ethanol concentrations affect the calcium leakage rate from the liposomes.

5.2.4. Mathematical description

A general equation describing diffusion from single-layered liposomes has been derived by Johnson and Bangham (1969). Fig. 20 shows a diagram of the system at a time t from the start of the leakage experiment. Liposomes with permeability p and volume v_c contain N - n counts. The dialysis bag has a permeability p_1, a volume v_1 and contains $n-n_2$ counts. The external solution has a volume v_2 and contains n_2 counts. Ideally, at the start of the dialysis experiment, all N counts should be in the liposomes. However, there may be some free

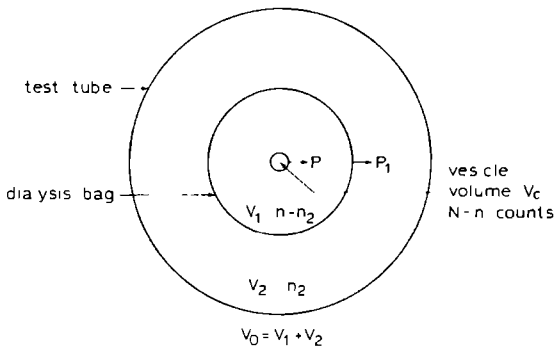


Fig. 20. Schematic presentation of the leakage experiments

counts in the dialysis bag released by the liposomes after leaving the Sephadex column but before start of the dialysis.
This number of counts is αN when t=0. Johnson and Bangham (1969) have derived the following general equation describing the leakage from the liposomes:

$$\frac{v_2}{v_o} - \frac{n_2}{N} = \frac{v_2}{v_o(K-L)} \left\{ (1-\alpha)Ke^{-Lt} - (L-\alpha K)e^{-Kt} \right\} \qquad (5\text{-}1)$$

where $v_o = v_1 + v_2$, $L = \frac{p}{v_c}$ and $K = \frac{p_1 v_o}{v_1 v_2}$.

When α is negligibly small, eqn. (5-1) becomes

$$\frac{v_2}{v_o} - \frac{n_2}{N} = \frac{v_2}{v_o(K-L)} \left\{ Ke^{-Lt} - Le^{-Kt} \right\} \qquad (5\text{-}2)$$

If the permeability of the dialysis bag is very much larger than that of the liposomes, then $K \gg L$ and eqn. (5-2) becomes

$$1 - \frac{n_2}{N} \frac{v_o}{v_2} = e^{-Lt} \qquad (5\text{-}3)$$

If we assume that $n_2 \cdot \frac{v_o}{v_2} = n$, the following simple relation results

$$1 - \frac{n}{N} = e^{-Lt} \qquad (5\text{-}4)$$

These relations have been derived for vesicles consisting of a single compartment surrounded by a single phospholipid bilayer, while our preparations consist of multi-layered liposomes. However, it will be shown below that these equations are applicable to such a system, by a simple change in the definition of L.

We assume that the lipid vesicles all have the same radius r_o and that each vesicle is composed of many concentric spheres of lipid bilayers (Fig. 21). The distance between two bilayers is always Δr.

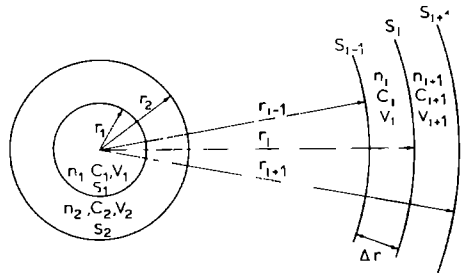


Fig. 21. Schematic presentation of leakage through multi-layered liposomes.

The radius of the i th sphere is r_i, its surface area S_i, the volume between S_i and S_{i-1} is v_i. This volume contains n_i cpm ^{45}Ca and the concentration of ^{45}Ca is c_i cpm/ml ($c_i = \frac{n_i}{v_i}$). Then:

$$S_i = 4\pi r_i^2 \tag{5-5}$$

$$v_i = 4\pi r_i^2 \quad r \tag{5-6}$$

The calcium efflux across the surface S_i is proportional to the concentration gradient $(c_i - c_{i+1})/\Delta r$ over this surface, and P is the permeability constant. Thus:

$$\frac{dn_i}{dt} = -PS_i \quad \frac{c_i - c_{i+1}}{\Delta} + PS_{i-1} \quad \frac{c_{i-1} - c_i}{\Delta r} \tag{5-7}$$

which, with $\Delta r \longrightarrow dr$ and $dc_i = c_{i+1} - c_i$, becomes

$$\frac{dn_i}{dt} = PS_i \quad \frac{dc_i}{dr} - PS_{i-1} \quad \frac{dc_{i-1}}{dr} \tag{5-8}$$

If $\frac{dc_i}{dr} = \frac{dc_{i-1}}{dr} + dr \frac{d^2 c_{i-1}}{dr^2}$,

then: $\frac{dn_i}{dt} = P\ 4\pi (r_{i-1} + dr)^2 \left\{ \frac{dc_{i-1}}{dr} + dr \frac{d^2 c_{i-1}}{dr^2} \right\} -$

$$- P\ 4\pi r_{i-1}^2 \quad \frac{dc_{i-1}}{dr} \tag{5-9}$$

If we neglect the higher order terms of dr, substitute eqn. (5-6) and leave out the suffixes:

$$\frac{dc}{dt} = \frac{2P}{r} \quad \frac{dc}{dr} + P \frac{d^2 c}{dr^2} \tag{5-10}$$

which is a diffusion equation. Its solution contains a Bessel function (Frank and v. Mises, 1943) and its approximate solution is given by

$$c = \frac{A}{\lambda r} \; e^{-\lambda^2 Pt} \sin(\lambda r) \qquad (5\text{-}11)$$

where A and λ are constant values. When $\lambda r = \pi = \lambda r'$, $c = o$. Thus

$$\lambda = \frac{\pi}{r'} \qquad (5\text{-}12)$$

The total counts in the vesicles are

$$l = j \int_o^{r_o} \frac{Ae^{-\lambda^2 Pt}}{\lambda r} \sin(\lambda r) \; 4\pi r^2 dr$$

$$= \frac{4\pi jA}{\lambda^3} \left\{ \sin(\lambda r_o) - \lambda r_o \cos(\lambda r_o) \right\} e^{-\lambda^2 Pt} \qquad (5\text{-}13)$$

where j is the number of vesicles. If $l = l_o$ ($\equiv N$) at t=o, then

$$l = l_o \; e^{-\lambda^2 Pt} \qquad (5\text{-}14)$$

When we use the notation of Johnson and Bangham (1969), eqn. (5-14) becomes

$$N-n = Ne^{-\lambda^2 Pt}$$

or $$1 - \frac{n}{N} = e^{-\lambda^2 Pt} \qquad (5\text{-}15)$$

This is the same equation as (5-4), only here $L = \frac{1}{v_c} p$ is substituted by $L = \lambda^2 P$.

5.3. RESULTS

5.3.1. Liposome formation

The procedure described in section 5.2.2 yields a suspension of liposomes, the membranes of which indeed enclose during their formation part of the radioactive calcium present in the medium. The sequestration of radioactivity by the liposomes, i.e. the percentage of the total

amount of radioactivity remaining in the lipid fraction after column elution, varies with the lipid composition. Liposomes derived from retinal lipids capture 12.8% (S.E.: 0.8, 15 determinations), phosphatidylcholine-phosphatidylserine (1:1) liposomes 4% (S.E.: 0.4, 2 determinations) and phosphatidylcholine liposomes 1% (4 determinations) of the total ^{45}Ca. Although considerable variations between different preparations of the same lipid composition may occur, due to a fairly random discarding of large (and thus much ^{45}Ca containing) liposomes, a clear difference in ^{45}Ca capture between the three types of liposomes remains. Variation in the calcium concentration in the medium, in which the liposomes are prepared, hardly affects the capture. No significant effect upon the percent ^{45}Ca which is sequestered by the liposomes, is

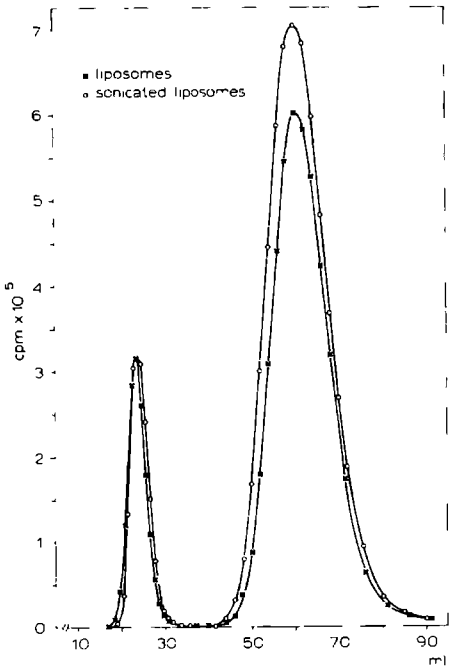


Fig. 22. Separation of retinal lipid liposomes from the radioactive medium by filtration over a Sephadex G-50 column. The liposomes are prepared in a ^{45}Ca containing medium (see methods) by mechanical shaking (-x-) or by mechanical shaking followed by sonication (-o-). After equilibration the suspension is layered on the column and eluted with the same - ^{45}Ca-free - buffer solution.

noted when the calcium concentration is varied between 0.005 and 10 mM.

The liposomes can be separated from the medium in which they have been prepared, by filtration over a Sephadex G-50 column. Fig. 22 shows that liposomal radioactivity and extra-liposomal radioactivity are clearly separated, both in suspensions prepared by shaking (this chapter) and by sonication (chapter 6).

5.3.2. Calcium efflux from liposomes

The calcium efflux from the three types of liposomes, treated as a first-order process by plotting log $(1 - \frac{n}{N})$ vs. time, is given in Fig. 23. The efflux rates from the phosphatidylcholine liposomes and the retinal lipid liposomes are about equal, the efflux from the phosphatidylcholine-phosphatidylserine liposomes is approximately 20 times faster. A control experiment without liposomes shows that the efflux rate from the dialysis bag is nearly 300 times as fast as that

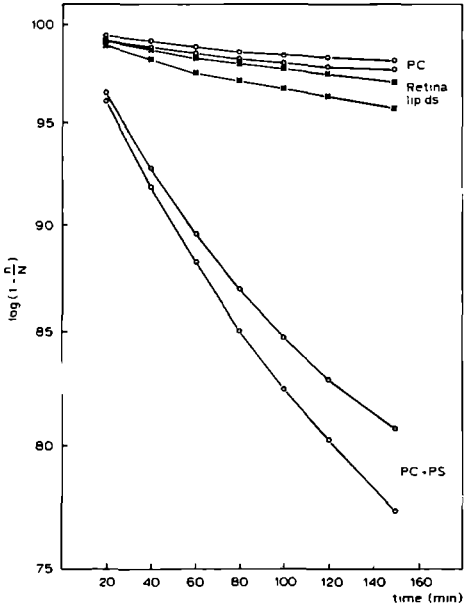


Fig. 23. ^{45}Ca efflux from different liposomes. Log $(1 - \frac{n}{N})$ is plotted against time (in min).

from both retinal lipid and phosphatidylcholine liposomes. This being the case, eqn. (5-4) will apply and the plot of log $(1 - \frac{n}{N})$ vs time should be linear. Only in the case of the phosphatidylcholine plus phosphatidylserine liposomes, where the efflux rate for the liposomes is rather close to that for the dialysis bag, the plotted curves deviate from the linear course expected on the basis of eqn. (5-4).

A striking phenomenon, which is shown more prominently for retinal lipid liposomes in Fig. 24, is the fact that the efflux curves for retinal lipid and phosphatidylcholine liposomes seem to be discontinuous: the curves are composed of two straight lines. The discontinuity in the curve usually occurs 60-100 min after the start of the dialysis experiment.

In most experiments the liposomes are prepared in a medium

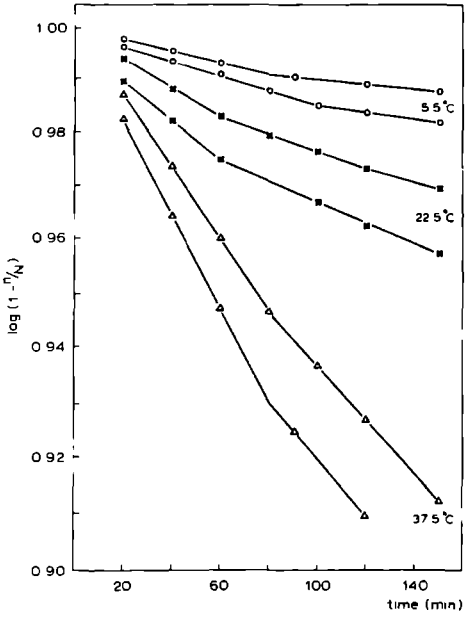


Fig. 24. ^{45}Ca efflux from retinal lipid liposomes. Log $(1 - \frac{n}{N})$ is plotted against time (in min) at three temperatures. Note that all lines are discontinuous.

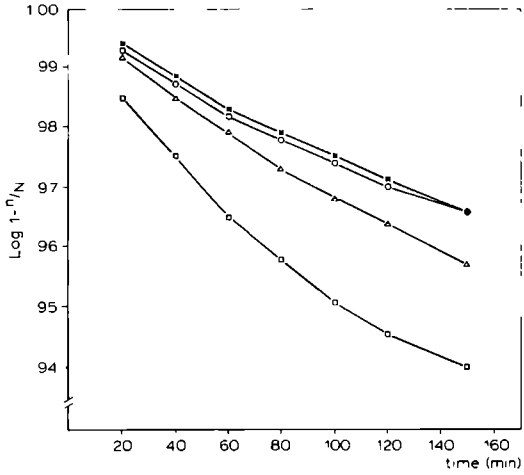


Fig. 25. ^{45}Ca efflux from retinal lipid liposomes prepared in media which contain different concentrations calcium: -x-, 10^{-2}M; -o-, 0.15 and 1 x 10^{-3}M; -Δ-, 0.5 x 10^{-4}M; -□-, 0.5 x 10^{-5}M. Log $(1 - \frac{n}{N})$ is plotted against time (in min).

containing 0.15 mM Ca^{2+}. When this concentration is varied between 0.005 and 10 mM, which should presumably change the calcium concentration inside the liposomes, a relatively small effect upon the efflux rates is noticed (Fig. 25). While decreasing the calcium concentration from 10 mM to 0.05 mM hardly effects the efflux, lowering the concentration a further 10-fold results in an increase in the efflux rate.

The efflux of calcium from retinal lipid liposomes has been measured at three temperatures, 5.5°C, 22.5°C and 37.5°C. Fig. 24 shows two typical efflux curves for each temperature. While experiments at the same temperature with different liposome preparations result in curves that do not completely coincide, due to differences in numbers of liposomes and in capture of ^{45}Ca, there is a much larger difference between leakage rates at the various temperatures. Moreover, in all experiments the leakage curve seems to be discontinuous. Fig. 26 presents the Arrhenius plots for both parts of the efflux curve. The

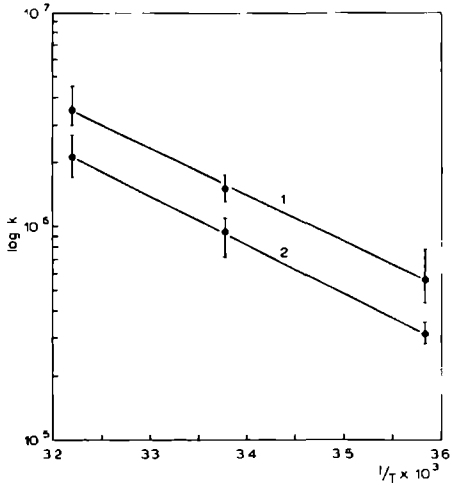


Fig. 26. Arrhenius plot for the ^{45}Ca efflux from retinal lipid liposomes.
1: early efflux period, activation energy 10.2 kcal/mol,
2: later efflux period (after discontinuity in efflux curve), activation energy 10.7 kcal/mol.

activation energies are 10.2 kcal/mol for the early part (0-80 min, Fig. 26-1) and 10.7 kcal/mol for the later part (80-150 min, Fig. 26-2), values which are not significantly different.

5.3.3. Effects of ionophores on calcium efflux

We have used two ionophores, which increase membrane permeability to biologically important alkali cations. The first one, X537A, forms complexes with both monovalent and divalent cations (Degani and Freedman, 1974) and transports them through biological membranes (Pressman, 1973). The second one, A23187, binds monovalent cations only weakly (Pfeiffer et al, 1974) and therefore acts as a much more specific divalent cation translocator (Reed and Lardy, 1972). They have both been added to retinal lipid liposomes. Both ionophores, especially when present in high cencentration, increase the calcium efflux rate to such an extent that it is not permissible to apply eqn. (5-4) to describe the efflux. Assuming, however, that $\alpha = 0$, we may apply eqn. (5-2) and plot $\log \left(\frac{v_2}{v_o} - \frac{n_2}{N}\right)$ against time. Typical results for experiments with X537A

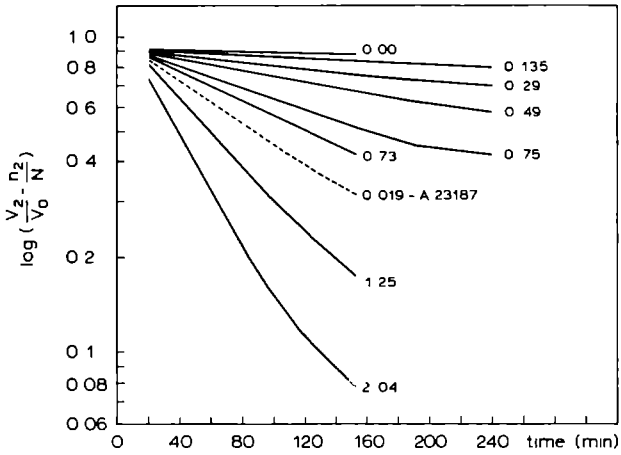


Fig. 27. ^{45}Ca efflux from retinal lipid liposomes in the presence of ionophore X537A. Log ($^{V}2/v_o$ - $^{n}2/N$) is plotted against the time t (eqn. (5-2)). Note that the ordinate scale is much larger than in Figs. 23-25. Seven different concentrations of X537A are used, while for comparison also one experiment with a relatively low concentration of A23187 is shown (broken line).

and A23187 are shown in Fig. 27, which indicates that increasing the X537A concentration from 0.1 to 2.0 µmol/l greatly increases the efflux rate. Ionophore A23187 is about 50 x as effective as X537A, as can be seen from the dotted curve in this figure.

Fig. 28 shows a comparison between an experimental curve and theoretical curves calculated from eqn. (5-2) for assumed values of L (7.2×10^{-5} min^{-1} and 8.0×10^{-5} min^{-1}) and the value of K (6.6×10^{-4} min^{-1}), both derived from measurements on the dialysis bag alone. The experimental curve deviates slightly upwards with time, which may be due to a non-uniform liposome size in the suspension. Eqn. (5-2) assumes that the fraction of counts (α) leaked out at t=0 is negligible, but in the presence of ionophore at t=0 min already a substantial amount of ^{45}Ca has leaked out. This introduces an error upon using eqn. (5-2), which however becomes much smaller after considerable time has elapsed. We have, therefore, taken the experimental values at t = 120 min to

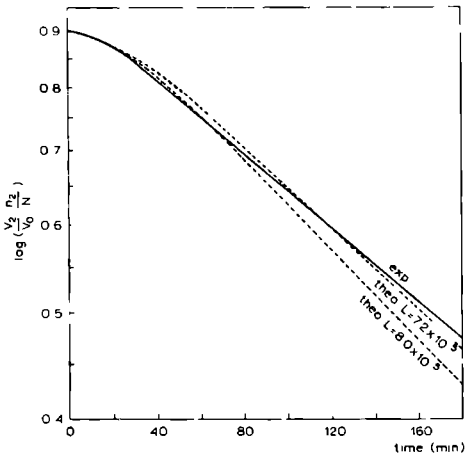


Fig. 28. Comparison between theoretical and experimental curves for the ^{45}Ca efflux from retinal lipid liposomes with X537A. Ionophore concentration: 0.75 µmol/l.

calculate the value of L for each ionophore concentration. Fig. 29 shows double-logarithmic plots of L (which is really a time constant, proportional to permeability) against the ionophore concentration. The different symbols represent the various liposome preparations. Also shown are theoretical lines expected for the three most likely types of permeant complexes of calcium with ionophore, indicating also the corresponding relation between L and the ionophore concentration c:

$$1\ Ca^{2+} + 2 \text{ ionophore molecules} : L=Ac^2 \quad (1:2)$$

$$1\ Ca^{2+} + 1 \text{ ionophore molecule} : L=Bc \quad (1:1)$$

$$1\ Ca^{2+} + 1 \text{ ionophore molecule} : L=Cc^{\frac{1}{2}} \quad (2:1)$$

where A, B and C are constant values. The 1:0 line represents the leakage of calcium in the absence of ionophore.

In the case of X537A (Fig. 29A) the calcium-ionophore complex apparently changes from a 1:1 ratio at low ionophore concentrations to

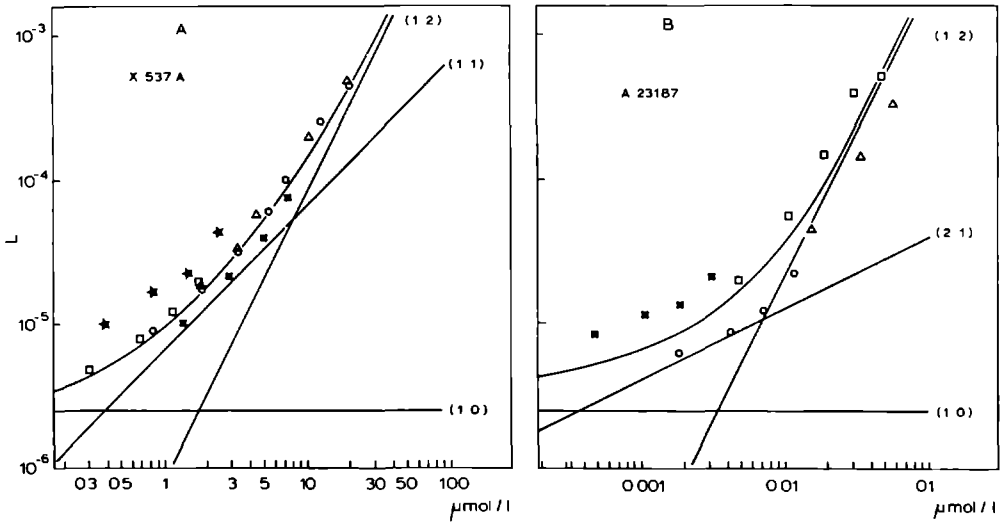


Fig. 29. Dependence of calcium permeability on ionophore concentration. Log L (L being proportional to the permeability) is plotted against log ionophore concentration (µ mol/l). The different symbols represent separate experiments.

A - ionophore X537A, experimental points, theoretical lines for one calcium ion interacting with 2 (1:2), 1 (1:1) and 0 (1:0) ionophore molecules and the curve resulting from summation of these three processes are shown.

B - ionophore A23187, experimental points, theoretical lines for one calcium ion interacting with 2 (1:2), $\frac{1}{2}$ (2:1) and 0 (1:0) ionophore molecules and the curve resulting from summation are shown.

a 1:2 ratio at high ionophore concentrations, since the curve resulting from the summation of these two lines (together with the 1:0 line) gives the best fit with the experimental points. In the case of ionophore A23187 (Fig. 29B) combination of another set of lines, viz. 1:0, 2:1 anc 1:2, gives the best-fitting curve. Thus it appears that A23187 at low concentrations transports two calcium ions per molecule, while this ratio changes at higher ionophore concentrations to one calcium ion per two ionophore molecules.

5.4. DISCUSSION

Most of our experiments have been carried out with liposomes made from retinal lipids in order to observe the behaviour of micelles with a lipid composition close to that of the natural rod disc membrane. A whole retina extract has been used, since it is easier to obtain than a rod disc extract, and the phospholipid composition of the retina (Anderson, 1970) is rather similar to that of the rod outer segment (Daemen, 1973). It is somewhat surprising that the ^{45}Ca capture in these retinal lipid liposomes (12.8%) is significantly higher than that in the phosphatidylcholine-phosphatidylserine type (4%) and the phosphatidylcholine type (1%). Since the phosphatidylcholine-phosphatidylserine ratio in retina is 4:1, it might be expected that retinal lipid liposomes are less negatively charged than the phosphatidylcholine-phosphatidylserine (1:1) type and thus capture less ^{45}Ca. However, as the opposite seems to be the case, it appears that the negatively charged serine residues of phosphatidylserine are not the main factor determining the calcium capture.

It is possible to calculate the partition of calcium ions between liposomes and medium. The liposome fraction after column elution contains about 10 mg lipid. If we assume that half of the liposome volume is water and the other half lipid, the total liposome volume should be about 20 µl. For retinal lipids this 20 µl contains about 10% of the total ^{45}Ca added. Before column separation of extra-vesicular ^{45}Ca the volume is 2 ml. If all calcium is equally distributed in the suspension, i.e. if no binding to lipids occurs, 20 µl should be expected to contain 1% of the radioactivity. This is indeed the case for phosphatidylcholine, which has no net negative charge and hence presumably offers less binding site for Ca^{2+} than e.g. phosphatidylserine. The fact that retinal lipid liposomes capture 12.8% of the calcium means that a large part of the sequestered Ca^{2+} ions must be bound to the lipid bilayer. This means that the partition coefficient

for Ca^{2+} between liposome and medium is 12.8.

It is also possible to calculate the free energy for capture of calcium. The loss in free energy upon capture of calcium is given by

$$\Delta G = - 2.303\ RT \log K_D \qquad (5\text{-}16)$$

(Knight, 1970), where K_D is the partition coefficient. Inserting a value of 12.8 for K_D, we obtain for ΔG a value of -1.5 kcal/mol. This low value would become somewhat larger, if we assume that the calcium ions are located on the polar head groups of the phospholipids. If these groups represent about 1/4 of the total liposome volume, the free energy difference would increase to -2.4 kcal/mol. This value is still too low for a direct chemical binding between a calcium ion and a negatively charged site, which would be of the order of 100 kcal/mol. Thus this suggests that Ca^{2+} is loosely and exchangeably bound to the phospholipid head group.

Fig. 23 indicates that the efflux rate varies with the lipid composition of the liposomes. Clearly phosphatidylcholine liposomes are rather impermeable for calcium ions. After two hours of dialysis only 2.2% (S.D.: 0.3, 3 determinations) of the calcium is lost from the liposomes. This value agrees rather well with that in an earlier report (Vanderkooi and Martonosi, 1971), where egg phosphatidylcholine liposomes have lost about 4% of their original calcium content after the same time of dialysis. However, the incorporation of phosphatidylserine in these liposomes enhances the leakage rate considerably. This is a well-known phenomenon, generally attributed to the net negative surface charge of phosphatidylserine. The relatively slow leakage rate from retinal lipid liposomes is more difficult to explain, since phosphatidylserine comprises 10-15% of these lipids. In addition, the degree of unsaturation of retinal lipids is considerably higher than in egg phosphatidylcholine (cf. chapter 6). Both factors should be expected (cf. de Gier et al, 1968) to enhance the leakage rate from retinal lipid liposomes as compared to that from pure phosphatidylcholine liposomes. The presence in retinal lipid extracts of a significant amount of

cholesterol, which comprises 13% of the phospholipid, might explain the discrepancy.

The efflux rate from retinal lipid liposomes is hardly affected by the calcium concentration in the medium in which they are prepared. Only when the calcium concentration is lowered under 0.05 mM, an increase in efflux rate is apparent (Fig. 25). This might be due to the fact that at calcium concentrations higher than 0.05 mM a certain amount of calcium is bound to the phospholipids. This bound calcium might inhibit the efflux through the bilayer of the captured calcium ions.

An as yet unexplained feature of the calcium leakage curve from retinal lipid liposomes (and in a lesser degree from phosphatidylcholine liposomes) is its biphasic character: each curve seems to be discontinuous and composed of two straight lines. Arrhenius plots of both parts of the curve give straight lines with nearly the same activation energies, 10.2 and 10.7 kcal/mol respectively. This activation energy is slightly lower than that for the leakage of univalent cations from phosphatidylcholine and phosphatidylcholine-phosphatidylserine liposomes, which amounts to about 15 kcal/mol (Papahadjopoulos and Watkins, 1967).

Since the Arrhenius plot forms a straight line, the retinal lipid liposomes apparently do not undergo a phase transition between 5°C and 37°C. The leakage vs. time curves, however, show a biphasic character. A tentative explanation can be offered along the following lines. Iizuka (1971) has shown that electric fields induce a high degree of orientation of liquid crystals of poly-$_{\gamma}$-benzyl-L-glutamate. Hence it could be that gel filtration of the liposomes on the Sephadex column yields an electric field as a result of a special distribution of Ca^{2+} ions in the liposomes. The liposomes would thus be forced into a certain conformation during the Sephadex filtration. This conformation could revert back to the original one during the leakage experiment, which transition would cause the discontinuity in the leakage curves.

It seems unlikely that the calcium efflux in our experiments would

be caused wholly or in large part by mechanical rupture of the liposomes. The following arguments against this possibility can be cited. Firstly, varying the rotation rate of the tubes during incubation over a 100-fold range (from 0.1 to 10 rpm) has no effect upon the efflux rates. Secondly, parallel experiments show that the efflux rate for sodium ions is consistently about 3 times higher than for calcium ions, while in case of mechanical rupture all ions should leak out at the same rate. Finally, the fact that both ionophores enhance the efflux of calcium is also an argument for the existence of closed liposomes. The possibility that the ionophores exert a lytic effect upon the liposomes is ruled out by the fact that in our experiments, at least A23187 does not enhance the sodium efflux rate.

Both ionophores, X537A and A23187, enhance calcium leakage from retinal lipid liposomes. However, X537A is much less effective than A23187: at relatively high ionophore concentrations, giving a 30-fold increase in calcium efflux rate, a 50-fold higher concentration of X537A is needed, while at low ionophore concentrations, sufficient to double the calcium efflux rate, a 125-fold higher concentration of X537A is required. We also find indications for a difference in the way in which the two ionophores transport calcium ions. In the presence of high ionophore concentrations a 1:2 Ca^{2+}-ionophore complex appears to exist for both ionophores, which is in agreement with some recent studies. Celis et al (1974), investigating the cation conductance in lipid bilayers, show evidence that in X537A mediated transport the 1:2 Ca^{2+}-ionophore complex is the permeant species. Likewise, both Case et al (1974) and Pfeiffer et al (1974), working with A23187, find indications for a 1:2 divalent cation-ionophore complex as a membrane carrier. Further evidence comes from crystallographic studies. Johnson et al (1970) find in a study of the barium salt of X537A that one barium ion, together with one molecule of water, is surrounded by two ionophore molecules in the unit cell, while the unit cell of cation-free A23187

also contains two ionophore molecules (Chaney et al, 1974).

At low ionophore concentrations we obtain evidence for the existence of two different complexes, a 1:1 Ca-X537A complex and a 2:1 Ca-A23187 complex. This finding may possibly be explained from the chemical structure of the two ionophores. X537A and A23187 have each one carboxyl and one carbonyl group, whereas A 23187 has in addition two ether-oxygen atoms, which might serve as ligands (Chaney et al, 1974). Hence, X537A appears to bind at most one Ca^{2+} ion and A23187 two Ca^{2+} ions.

The experiments with the two ionophores indicate that the very low calcium permeability of phospholipid bilayers can be increased very considerably (100-fold or more) by the presence of carrier-like substances in the membrane. In this respect, it is interesting to note that Martonosi et al (1974) have reported that incorporation of purified calcium transport ATPase into phospholipid liposomes, both substances derived from sarcoplasmic reticulum, greatly increases the passive diffusion of calcium through the liposome membrane. Possibly, the protein complex acts as a 'fixed' carrier for the calcium ions. It would be interesting to see whether incorporation of rhodopsin into phospholipid liposomes would have a similar effect.

CHAPTER 6

MOVEMENT OF SODIUM IONS THROUGH ARTIFICIAL LIPID MEMBRANES

6.1. INTRODUCTION

The diffusion of sodium ions through membranes of the photoreceptor cell apparently is an important process in visual function. Sodium ions are thought to be the charge carriers of the current which enters the outer segments in the dark. Illumination decreases this sodium influx, presumably by the release of calcium ions which close the sodium channels (cf. Fig. 8). These processes occur at the rod outer segment outer membrane.

We have examined the sodium permeability of the basic phospholipid bilayer structure of the rod outer segment membranes, again using liposomes as a model system (cf. chapter 5). As the disc membranes originate from invaginations of the cell membrane, it seems reasonable to assume that the lipid composition of the outer membrane closely resembles that of the rod disc membrane. Therefore, the sodium permeability characteristics of liposomes formed from disc membrane lipids, which lipids constitute the bulk of the total outer segment lipid material, may reflect the permeability properties of the phospholipid bilayer of the outer membrane.

In order to assess whether a bilayer formed from rod outer segment lipids shows exceptional behaviour with respect to its sodium permeability, we have compared the sodium leakage from liposomes composed of rod outer segment lipids, retinal lipids, phosphatidylcholine and phosphatidylcholine-phosphatidylserine mixtures. We have also investigated whether the presence of calcium ions can influence the leakage of sodium ions through liposomes.

6.2.1. Lipid extraction

Lipid is extracted from whole cattle retina as described in section 5.2.1.

Rod outer segments are isolated using a sucrose density gradient as described in section 2.2.1. The rhodopsin containing layer is collected and diluted with one volume 0.16 M Tris-HCl, pH 7.4. After centrifugation (10 min at 4600 x g) the sediment is washed once with water, and lipid is extracted from the particulate material using a chloroform-methanol mixture as described for the extraction of retinal lipids (section 5.2.1).

Phosphatidylcholine and phosphatidylserine are isolated as described in section 5.2.1.

All lipids are dissolved in benzene-ethanol (4:1) and stored at $-20^{\circ}C$ under N_2.

6.2.2. Liposome preparation

Liposomes are prepared in the following way. Organic solvent is removed from 30 mg of phospholipid in a rotating evaporator. To the resulting thin film is added 4 ml of a salt solution containing: NaCl, 135 mM; Tris-HCl (pH 7.4), 10 mM and 3-6 µCi of ^{22}Na (3.5 Ci/mmol, Radiochemical Centre, Amersham, Nottingham, England). In some experiments with ^{45}Ca mentioned in this chapter, 0.1 mCi ^{45}Ca (445 mCi/mmol, Radiochemical Centre, Amersham) is added instead of ^{22}Na, which brings the calcium concentration in the medium to approximately 0.05 mM. Suspension is achieved by vigorous mechanical shaking of the mixture under N_2 for 30 min. The suspension is then sonicated using a Branson B12 sonifier with microtip used at half maximal output. The material is cooled in ice and sonicated under N_2 for 10 x 1 min with 1 min intervals. The suspension is then left overnight at $4^{\circ}C$ to equilibrate.

The removal of radioactive ions which have not been trapped in the liposomes is as described in section 5.2.2.

6.2.3. Leakage experiments

Samples of 1 ml of suspension are placed in small dialysis bags (Visking tube, boiled in a solution containing 2 mM $NaHCO_3$ and 0.2 mM EDTA and thoroughly washed with double-distilled water) and the closed bags are placed in test tubes (1.3 x 14.5 cm) containing 12 ml of either 135 mM NaCl, 10 mM Tris-HCl (pH 7.4) or 145 mM Tris-HCl (pH 7.4). The stoppered tubes are attached to a vertically rotating disc (7 rev/min). Over a dialysis period of at least 80 min, 1 ml samples for radioactive counting are taken from the outer solution and at the end of the experiment the radioactivity remaining inside the dialysis bag is also measured.

All radioactive samples are dissolved in 10 ml Aquasol (New England Nuclear) and counted in a Philips liquid scintillation analyzer.

6.2.4. Analytical methods

The lipid phosphorus in the lipid extracts is determined using a modified Fiske-Subbarow method after H_2SO_4-$HClO_4$ digestion (Broekhuyse, 1968). In calculating the phospholipid content the average phosphorus content of the phospholipids is assumed to be 4%.

Cholesterol is quantitatively determined after saponification of the lipid extract with alcoholic KOH. Aliquots of these extracts containing 20-180 µg cholesterol are subjected to analysis by a modified Liebermann-Burchard reagent (Abell et al, 1952).

Preparation of fatty acid methyl esters for gas-liquid chromatography is carried out with borontrifluoride by the method of Morrison and Smith (1964). Gas chromatographic separations are performed on an ethylene glycol succinate column at 200°C (Broekhuyse, 1972). For identification purposes a silicone column (0.125 inch x 6 ft) is used, packed with 3% SE-30 on Gas-chrom Q (100-120 mesh). The carrier gas is

N_2 (15 ml per min), the temperature is isothermal 200°C or is linearly programmed from 160-200°C (2.5°C per min).

For electron microscopy the negative staining technique is used. The liposomes are prepared as described in section 6.2.2, but without radioactive ions. A small drop of the suitably diluted suspension is placed on the surface of a carbon film on a copper grid. Excess liquid is removed with a capillary pipette. Then one drop of a 3% solution of ammonium molybdate (pH 6.3, 300 mOsm) is placed on the grid and again the excess liquid is removed. Electron micrographs are recorded with a Philips EM300 or EM301 microscope, operated at 60 kV, and using a specimen chamber cooling device.

6.3. RESULTS

6.3.1. Liposome formation

Treatment of phospholipids according to the method described in section 6.2.2, yields a suspension of lipid vesicles which sequester part of the radioactive sodium present in the medium. The dry phospholipids are mechanically shaken in the buffer solution and then sonicated in an attempt to obtain a relatively uniform particle size distribution. While pure phosphatidylcholine, the phosphatidylcholine-phosphatidylserine mixture and the retinal lipids are easily suspended in the medium by mechanical shaking and yield a clear solution, the rod outer segment lipids behave differently in this respect. Here, the addition of two small glass beads is necessary to promote dispersion of the lipid from the wall of the flask and longer mechanical shaking is needed to obtain a clear solution. In this case some lipid material usually remains unsuspended, even after as long as 2 h of mechanical shaking. After sonication the liposomes can be separated from the medium, in which they have been prepared, by gel filtration in the same way as non-sonicated liposomes (Fig. 22).

The capture of sodium ions in the different types of liposomes is

Table XIV. Capture of radioactive ions by various types of liposomes. The liposomes are prepared as described under Methods and separated from their original medium by filtration over a Sephadex G-50 column. Results are expressed as percent of total radioactivity eluted from the column, present in the liposomal fraction (with standard errors of the mean).

	^{22}Na	^{45}Ca
Phosphatidylcholine	0.6 ± 0.2 (2)	1.0 (4)*
Phosphatidylcholine-phosphatidylserine (4:1)	1.2 ± 0.1 (3)	-
Retinal lipids, sonicated	1.9 ± 0.3 (4)	-
Retinal lipids, non-sonicated	5.2 ± 1.7 (2)	12.8 ± 0.8 (15)
Rod outer segment lipids	0.7 ± 0.3 (4)	1.8 ± 0.8 (2)

* prepared without sonication

represented in Table XIV. For purposes of comparison, the data for the capture of calcium are added. It can be seen that rod outer segment lipid liposomes capture less ^{22}Na (0.7%) than liposomes derived from retinal lipids (1.9%). Moreover, sonication decreases the capture, as is shown by the values for the capture of ^{22}Na by retinal lipid liposomes. The sonicated vesicles capture 1.9% of the ^{22}Na present in the medium, while the non-sonicated liposomes sequester 5.2% of the total radioactivity. This is also true for the capture of ^{45}Ca by the same type of liposomes, as is shown in Fig. 22.

Factors which are important in determining the ion capture and the leakage rate are the size and the structure of the liposomes. The size, shape and general configuration of the liposomes depend on the particular phospholipid used, as well as on the ionic strength, the valency of the ionic species and the pH of the aqueous medium. In our experiments all liposome types are formed in the same medium. The use of different phospholipids, however, makes it probable that there exist

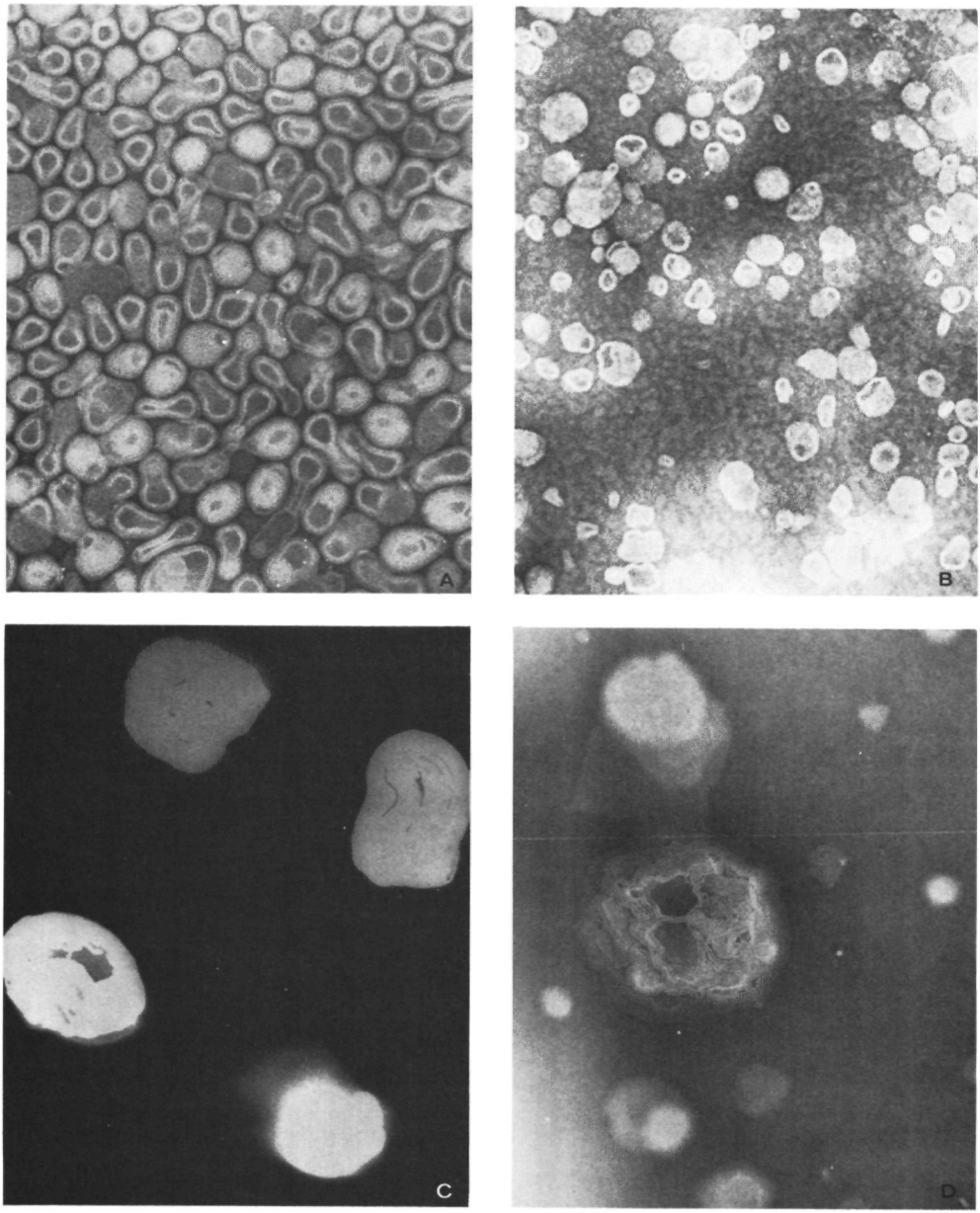


Fig. 30. Electronmicrographs of negatively stained liposomes. Liposomes are prepared as described under Methods and stained with ammonium molybdate. A: phosphatidylcholine liposomes (120 000 x); B: phosphatidylcholine-phosphatidylserine (4:1) liposomes (115 000 x); C: rod outer segment lipid liposomes (72 000 x); D: retinal lipid liposomes (69 000 x).

differences in structure between the various liposomal types, e.g. in size of the central compartment and in number of surrounding bilayers. We have examined the morphology of the various liposomes under the electron microscope by means of a negative staining technique. Sonicated phosphatidylcholine liposomes form mostly single-walled vesicles of a relatively uniform size (diameter ca. 0.06 μ, Fig. 30A), while non-sonicated phosphatidylcholine liposomes have a much larger central compartment surrounded by a large number of bilayers (not shown). The phosphatidylcholine-phosphatidylserine mixture yields a somewhat analogous suspension of liposomes (Fig. 30B), having about the same size as those formed from phosphatidylcholine alone. However, the pictures obtained from rod outer segment lipid liposomes show much larger particles (diameter ca. 0.3 μ) which are hardly penetrated by the negative stain (Fig. 30C). Negative staining of the retinal lipid liposomes does not yield an uniform picture. Mostly only large arrays of membranes can be seen and occasionally vesicle-like structures as in Fig. 30D. However, there are good reasons for assuming that retinal lipid liposomes form completely sealed particles: the leakage rate of sodium is higher than the leakage rate of calcium, ionophores greatly enhance the efflux rate of calcium and the presence of EGTA (a calcium chelating agent) in the dialysis medium does not affect the calcium efflux from these liposomes.

Thus it should be emphasized that the negative staining technique imposes rather harsh conditions upon the samples during preparation. Therefore, it is difficult to decide whether the apparent differences in morphology between the various liposomal preparations reflect native structural differences in the liposomes or differences induced by the negative staining technique.

6.3.2. Sodium efflux from liposomes

The sodium efflux from the four types of liposomes is given in Fig. 31. In general the efflux rates are too high to apply eqn. (5-2)

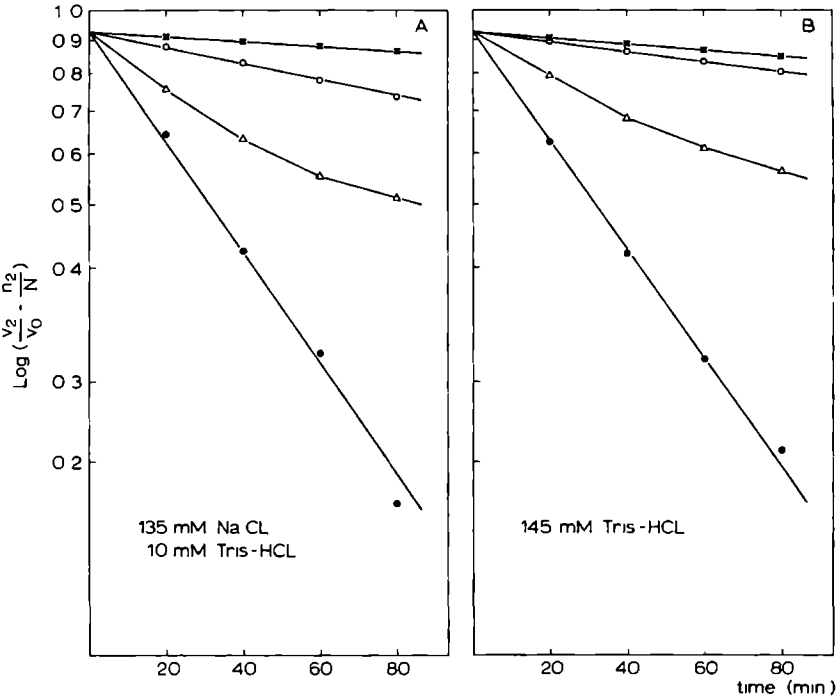


Fig. 31. Efflux of ^{22}Na from different types of sonicated liposomes. Log ($^{v}2/v_o - {}^{n}2/N$) is plotted against time (in min). One ml liposome suspension (in 135 mM NaCl, 10 mM Tris-HCl, pH 7.4) is dialyzed against 12 ml 135 mM NaCl, 10 mM Tris-HCl, pH 7.4 (A) or 12 ml 145 mM Tris-HCl, pH 7.4 (B). The liposomes are composed of phosphatidylcholine (-x-), retinal lipids (-o-), a 4:1 phosphatidylcholine-phosphatidylserine mixture (-Δ-) or rod outer segment lipids (-•-).

to describe the efflux. Therefore, we have applied eqn. (5-4) and plotted log ($^{v}2/v_o - {}^{n}2/N$) against time. Two dialysis media have been used, 135 mM NaCl, 10 mM Tris-HCl, pH 7.4 (Fig. 31A) and a medium without sodium, containing only 145 mM Tris-HCl, pH 7.4 (Fig. 31B). The behaviour is the same in both media. All efflux curves are reasonably straight lines with exception of those from phosphatidylcholine-phosphatidylserine liposomes, which are clearly discontinuous.

Phosphatidylcholine liposomes show a relatively low efflux. In the first hour of dialysis 1.8% of the total sequestered sodium leaks out. This value comes close to that for ^{42}K leakage from the same type of liposomes, 0.5%/h, as reported by Papahadjopoulos and Watkins (1967). The efflux from retinal lipid liposomes, measured after 30 min dialysis,

is 2-3 x higher. Liposomes composed of a 4:1 phosphatidylcholine-phosphatidylserine mixture, which is the same phospholipid ratio as is found in retina (Anderson, 1970) and rod outer segments (cf. Daemen, 1973), lose sodium 7-11 times as fast as the phosphatidylcholine liposomes. Liposomes composed of rod outer segment lipids are even more leaky, losing sodium 18-22 times faster than phosphatidylcholine liposomes and 7-13 times faster than the retinal lipid liposomes. This last difference is particularly interesting since there is little difference in phospholipid composition of whole retina and rod outer segments.

The high sodium leakage rate from rod outer segment lipid liposomes raises the question whether this lipid mixture forms incompletely sealed particles, with ions diffusing through large openings between lamellae rather than across continuous bilayers. This phenomenon is known to occur with pure phosphatidylethanolamine (Papahadjopoulos and Watkins, 1967). If this should be the case, no discrimination between the leakage of different ions is expected. Fig. 32, however, shows that there exists a clear difference between the leakage rates of sodium and calcium from liposomes formed from rod outer segment lipids, the leakage rate for ^{22}Na being about 4 times faster. A similar discrimination is made by retinal lipid liposomes, which lose ^{22}Na twice as fast as ^{45}Ca. Thus it appears that both types of liposomes do indeed form closed structures. This is further indicated by the fact that addition of the calcium chelating agent EGTA (1mM) to the dialysis medium does not enhance the calcium efflux from these liposomes. In 'open' structures, where calcium can be reached by a relatively large molecule as EGTA which is unable to pass a lipid bilayer, an effect would have been expected.

6.3.3. Fatty acid composition of lipid extracts

The spectacular difference in sodium leakage rate between liposomes composed of retinal and of rod outer segment lipids raises the question which factors are responsible for this phenomenon. Since the phospholipid

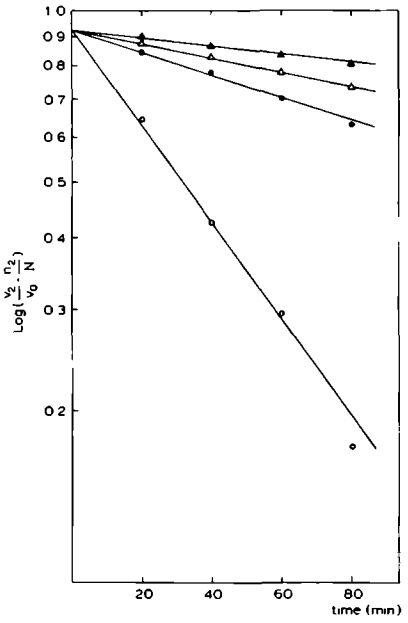


Fig. 32. Efflux of ^{22}Na and ^{45}Ca from sonicated liposomes, composed of retinal and rod outer segment lipids. One ml liposome suspension is dialysed against 12 ml 135 mM NaCl, 10 mM Tris-HCl, pH 7.4. The leakage rates from retinal lipid liposomes (^{22}Na, -Δ-; ^{45}Ca, -▲-) and rod outer segment lipid liposomes (^{22}Na, -o-; ^{45}Ca, -●-) are plotted as log ($^{\nu}2/v_o$ - $^{n}2/N$) vs time (in min).

composition of whole retina is about the same as that of isolated rod outer segments, it appears unlikely that variation in phospholipid composition is the source of the difference in leakage rate.

However, another important factor which influences the permeability properties of the liposomes is the fatty acid composition of the lipids (de Gier et al, 1968). Rod outer segments are known to contain a high amount of poly-unsaturated fatty acids (cf. Daemen, 1973). Therefore we have compared the fatty acid composition of the lipids which constitute the different types of liposomes (Table XV). There indeed exists a great difference in degree of unsaturation of the fatty acid chains between the lipid extracts from retina and rod outer segments: the

Table XV. Fatty acid composition of lipids used for liposome preparation. The major fatty acids are expressed as percentage of the total fatty acid content.

Fatty acid (C atoms: double bonds)	Phosphatidyl choline	Phosphatidyl serine	Cattle retina extract (2)	Cattle rod outer segment extract (3)
16:0	1.2	52.0	20.2 ± 0.5	13.0 ± 0.9
16:1	0.1	0.8	1.0	0.4
18:0	37.2	10.1	19.9 ± 0.9	19.3 ± 0.2
18:1	31.2	19.0	10.7 ± 0.2	3.2 ± 0.3
18:2	-	11.5	1.4	0.7 ± 0.1
20:4	1.6	2.9	9.7 ± 0.7	5.4 ± 0.7
22:4/22:5	5.2	0.4	7.4 ± 2.2	8.2 ± 3.4
22:6	7.2	2.5	24.1 ± 3.5	48.2 ± 4.4

calculated number of double bonds per mole fatty acid is 2.3 for retinal lipids and 3.5 for rod outer segment lipids. Particularly noteworthy is the difference in docosahexaenoic (C22:6) acid content, which is in rod outer segment lipids twice as high as whole retina lipid extracts. Moreover, the percentage of this long-chain, highly unsaturated fatty acid species is even higher than previously reported for rod outer segment extracts (cf. Daemen, 1973).

The cholesterol content of the lipid extracts has also been reported to be an important factor controlling the permeability of liposomes (de Gier et al, 1968; Demel et al, 1972). Therefore we have determined the amount of cholesterol in our lipid extracts. When expressed as weight-percent of the phospholipid content, the retinal lipid extracts contain 13.4% (S.D.: 0.4, 2 determinations) and the rod outer segment lipid extracts 4.3% (S.D.: 1.7, 3 determinations) cholesterol.

Table XVI. Effect of calcium on ^{22}Na leakage from various types of sonicated liposomes. One ml liposome suspension is dialysed against 12 ml buffer, with or without 5 mM calcium. The results in column 2 and 3 are expressed as the percentage of captured ^{22}Na, which is lost during 30 min dialysis. A: dialysis medium 145 mM Tris-HCl, pH 7.4; B: dialysis medium 135 mM NaCl-10 mM Tris-HCl, pH 7.4.

	Liposome composition	- Ca	+ Ca	Ratio	No of experiments	Total no of determinations	P value *
A	Phosphatidyl choline	3.3	1.2	0.36	1	6	0.10
	Phosphatidyl choline-phosphatidyl serine (4:1)	20.5	16.9	0.82	2	12	0.09
	Retinal lipids	5.2	4.2	0.81	3	16	0.47
	Rod outer segment lipids	41.1	39.6	0.96	2	12	0.70
B	Phosphatidyl choline	1.1	0.8	0.73	1	6	0.10
	Phosphatidyl choline-phosphatidyl serine (4:1)	27.9	23.1	0.83	2	10	0.22
	Retinal lipids	7.2	4.6	0.64	3	16	0.005
	Rod outer segment lipids	37.4	40.8	1.09	2	8	0.20
						Combined test:	< 0.001

* Determined by the Wilcoxon test

6.3.4. Effect of calcium on sodium leakage from liposomes

It has been proposed that calcium acts in visual excitation by closing the sodium channels in the rod outer segment outer membrane. We have investigated whether addition of calcium ions to the dialysis medium affects the leakage rate of sodium ions from the various types of liposomes. Table XVI represents the results of our experiments. Only in one case does calcium have a significant effect. Addition of 5 mM calcium to a dialysis medium containing 135 mM NaCl, 10 mM Tris-HCl, pH 7.4, decreases the leakage rate of ^{22}Na from retinal lipid liposomes. However, the same phenomenon does not occur in a medium which contains only 145 mM Tris-HCl.

In neither of the two media does calcium significantly affect the sodium leakage from rod outer segment lipid liposomes. We have also tried to sequester calcium inside this type of liposome to see whether this would influence the sodium leakage. It appears, however, impossible to prepare liposomes from rod outer segment lipids in the presence of 5 mM calcium. Even when only 0.5 mM calcium is added to the medium in which the liposomes are usually formed, no clear lipid suspension can be obtained. This is the more intriguing because addition of calcium, up to a concentration of 10 mM, does not prevent the formation of liposomes from retinal lipids.

However, when all experiments with the various liposome types in both media are regarded together, a significant ($P < 0.001$) effect of calcium is apparent: the sodium leakage is decreased by the presence of calcium ions.

6.4. DISCUSSION

It seems reasonable to assume that the phospholipid mixtures used for the work described in this chapter do indeed form closed lamellar structures. While this has already been shown for liposomes composed of pure phosphatidylcholine (Papahadjopoulos and Miller, 1967), the phosphatidylcholine-phosphatidylserine mixture and the lipid mixtures

extracted from rod outer segments and whole retina appear to behave similarly. A first indication is given by the electronmicrographs, which always show closed structures, except in the case of retinal lipid liposomes. Furthermore, for retinal lipid and rod outer segment lipid liposomes this is strongly indicated by the fact that the sodium leakage rate from both types of liposomes is significantly higher than the calcium leakage rate (Fig. 32). Another indication is given by the fact that the presence of EGTA in the dialysis medium does not affect the efflux of calcium.

The size and shape of the liposomes are dependent on the phospholipid species used. This appears to be illustrated by the electronmicrographs of negatively stained liposome preparations. The size of the different liposomes, which are all sonicated for 10 min, varies considerably. Phosphatidylcholine liposomes are small with a relatively uniform size distribution, their diameter being about 0.06 μ. This agrees rather well with the phosphatidylcholine liposomes described by Papahadjopoulos and Miller (1967), which have a diameter of ca. 0.08 μ. While the liposomes composed of phosphatidylcholine plus phosphatidylserine have about the same size, those composed of rod outer segment or retinal lipids are larger (diameter ca. 0.3 μ). They also show many concentric membranes while the negative stain hardly seems to penetrate the liposomes composed of rod outer segment lipids. It has already been pointed out that the technique used for preparation of the samples for electron microscopy imposes rather harsh conditions on the structures. There is no guarantee at all that the final pictures represent the native situation, and therefore not too much emphasis must be placed on these results.

It appears likely that the four types of liposomes differ in size and shape. This makes a direct comparison of the sodium efflux rates as a function of the phospholipid composition difficult, because differences in size and shape of the liposomes may affect the efflux rates. However, it appears that these variations in size do not change

the efflux rates too drastically. Papahadjopoulos and Watkins (1967) have compared the leakage rates of some ions from various liposomes, which had been prepared by manual shaking, mechanical shaking or sonication. Sonication produces small vesicles with one or two concentric bilayers, while shaking yields much larger structures surrounded by many bilayers. While a high efflux rate might be expected for smaller liposomes, due to a high surface to volume ratio, the opposite is true. The small vesicles produced by sonication generally show about half as high an efflux rate as the liposomes produced by mechanical shaking. This finding is confirmed in our experiments with phosphatidylcholine-phosphatidylserine liposomes. The sonicated liposomes lose 33% of their ^{22}Na during 1 h of dialysis, while those prepared by mechanical shaking lose 45% sodium per h. The sodium efflux from sonicated retinal lipid liposomes, on the contrary, is 13% per h, twice as high as the efflux from the non-sonicated preparation (6% per h). Thus it appears that, while changes in size and structure may affect the efflux rates, the differences are not as large as those encountered between for instance retinal lipid and rod outer segment lipid liposomes.

The difference in sodium leakage rates for retinal lipid liposomes and liposomes composed of rod outer segment lipids indeed is spectacular. Rod outer segment lipid liposomes in NaCl-Tris lose sodium 7 times and those in Tris 13 times faster. In view of the foregoing discussion, it appears that this difference must be explained by the composition rather than the configuration of the liposomes. Assuming the phospholipid composition of both preparations to be about the same (Anderson, 1970; Daemen, 1973), there must be other factors responsible for this difference.

The presence of large amounts of cholesterol is known to decrease the permeability of liposomes for glycerol (de Gier et al, 1968; Demel et al, 1972) and cations (Scarpa and de Gier, 1971; Papahadjopoulos et

al, 1972). There is indeed a difference in cholesterol content between our lipid extracts; in the rod outer segment extract cholesterol constitutes 4.3% of the total phospholipid weight, in the retinal extract 13.4%. This additional 10% cholesterol seems, however, insufficient to explain a large decrease in permeability, because addition of 10% cholesterol to synthetic lecithins hardly decreases their permeability (de Gier et al, 1968; Demel et al, 1972). To make a direct comparison, we have tried to prepare liposomes of rod outer segment lipids containing an additional 10% cholesterol. This mixture, however, does not yield a clear liposome suspension.

The saturation of the fatty acid chains is another important factor controlling the permeability of liposomes (de Gier et al, 1968; Moore et al, 1969; Scarpa and de Gier, 1971; Chen et al, 1971). The fatty acids of the rod outer segment membranes are highly unsaturated and this is in our opinion the factor which mainly determines the permeability difference between retinal and rod outer segment lipid liposomes. The data in Table XV show that the fatty acids in our rod outer segment extracts are far more unsaturated than those in the retinal extract. This is mainly caused by the extremely high docosahexaenoic acid content (48%) in the former. This percentage is significantly higher than previously reported for rod outer segment membrane extracts, which range from 23-37% (cf. Daemen, 1973). This warrants a more thorough examination of the fatty acid composition of cattle rod outer segment membranes, since the purification methods have been improved after the publication of the earlier, lower values.

Finally, we have investigated whether the presence of 5 mM calcium in the dialysis medium has any effect on the sodium leakage rate. Our main interest has been to examine whether the permeability properties of the phospholipid part of the rod outer segment membranes can be influenced by calcium ions. The tentative conclusion from our results (Table XVI) appears negative: neither in NaCl-Tris nor in Tris alone

does addition of calcium significantly affect the sodium leakage rate from rod outer segment lipid liposomes. Thus it appears that protein rather than lipid components of rod membranes mediate the proposed calcium induced decrease in sodium influx.

A significant decrease is shown by the retinal lipid liposomes in a Tris-HCl medium. The reason why calcium has no significant effect on the permeability of the same liposomes in a NaCl-Tris medium remains unclear. While an effect of calcium is certainly indicated in the case of phosphatidylcholine liposomes, the total number of determinations is as yet too small to speak of a really significant effect. The same, though to a lesser degree, holds for the phosphatidylcholine-phosphatidylserine liposomes. If we apply the Wilcoxon test to the combined results of all experiments, it indeed appears that calcium significantly reduces the sodium permeability of the liposomes.

It seems obvious that a possible effect of calcium is closely related to the degree of calcium binding by the different liposome preparations. Therefore, if retinal lipid liposomes can be 'closed' by calcium ions, presumably by their binding to the phospholipid head groups, it might be expected that rod outer segment lipid liposomes, composed of the same phospholipids, are affected in a similar fashion. The absence of an effect on rod outer segment lipid liposomes might be interpreted to mean that here less calcium is bound to the phospholipids. This again could be caused by the high percentage of unsaturation in the fatty acid chain, since Shah and Schulman (1967) find that introduction of unsaturated fatty acids in lecithins decreases their affinity for divalent cations.

In conclusion it can be stated that the presence of an exceptionally high degree of unsaturation in the fatty acid moiety introduces a high sodium (and calcium) permeability in liposomes composed of rod outer segment lipid extracts, as compared to retinal lipid liposomes. This property of the lipid core of the rod outer segment

membranes may be responsible for the high sodium permeability of these membranes in *vivo*. It would be interesting to observe whether incorporation of rhodopsin in rod outer segment lipid liposomes further increases their sodium permeability and possibly even introduces a 'calcium sensitivity' of the sodium leakage.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7.1. INTRODUCTION

All experiments described in this thesis have been performed, directly or indirectly, to elucidate the important question: which substance acts in visual excitation as the transmitter between rod disc and rod outer membrane ?

At the time we started our experiments, both cAMP and calcium had been proposed to function in this process. Therefore we have investigated the presence of cAMP and calcium in vertebrate rod outer segments and whether light in any way can change either the concentration or the intracellular distribution of these two compounds. Furthermore, as a first step to model membranes which contain rhodopsin, we have examined the permeability properties of artificial lipid vesicles (liposomes).

In this chapter we shall briefly recapitulate the most important results of our studies and discuss them in connection with relevant results of other workers. Suggestions for possible future experiments will be made.

7.2. CYCLIC AMP.

Since the time we started looking for the presence in photoreceptors of adenylate cyclase, the enzyme which forms cyclic AMP, it has become increasingly clear that the very high specific activities reported to be present in rod outer segments (Bitensky et al, 1971a,b; Miller et al, 1971) are in fact a 100-fold lower. Not only our results, but also those of other groups indicate that the true specific activity of adenylate cyclase in both frog and cattle rod outer segment suspensions, isolated and assayed in the dark, ranges from 0.2-0.5 nmol cyclic AMP/mg protein per 10 min (Table V). Moreover, the results of our fractionation

experiments (Table II) and those of Zimmerman (Fig. 10) show the presence of another retinal fraction, which contains an adenylate cyclase with a much higher specific activity than is present in rod outer segments. Thus it appears not altogether impossible that even the relatively low adenylate cyclase activity in our outer segment suspensions originates in fact from other retinal structures.

Light affects the adenylate cyclase activity, present in outer segment preparations (Fig. 11). This is not a real argument that the enzyme activity originates from the outer segments, since we have shown that light can inhibit exogeneous adenylate cyclase, which is added to a suspension of rod outer segments (Table IV). In our opinion light releases a diffusible factor, which can inhibit the adenylate cyclase present (or possibly stimulate a phosphodiesterase). It is tempting to propose that calcium ions may be responsible for this phenomenon, since calcium clearly inhibits the adenylate cyclase activity (Table III), even though addition of EGTA before illumination does not decrease the light-inhibition.

In conclusion, we may state that it is highly questionable whether the adenylate cyclase activity found in rod outer segment suspensions does indeed originate from these cells. It appears that the adenylate cyclase activity decreases with increasing purity of the outer segment suspension. In any case, a role of the enzyme in visual excitation, as proposed by Bitenski et al (1971, 1972a,b), seems improbable. It is entirely unclear which function cAMP, if present in rod outer segments at all, would perform. Cyclic AMP does not appear to affect the dark or light-stimulated phosphorylation of rhodopsin (Kühn and Dreyer, 1972; Frank et al, 1973; Weller et al, 1975), nor does it influence the time which an isolated frog retina needs to recover its light sensitivity (Hood and Ebrey, 1974).

Therefore, there exists at this moment no evidence for a role of cAMP in photoreceptor function.

7.3. CALCIUM

The hypothesis that calcium ions play a transmitter role in visual excitation is a fairly recent one (Yoshikami and Hagins, 1971; Hagins, 1972). This proposal is based on electrophysiological evidence, mainly the effects of variations in the external calcium concentration on the dark- and photocurrents in rod outer segments (cf. Yoshikami and Hagins, 1973). We have tried to collect evidence to support this hypothesis. The results of our experiments are described in chapters 3 and 4.

The first requirement which must be met for this hypothesis to be valid is obviously that calcium ions are present in rod outer segments in high concentration. Although it cannot be excluded that the isolated frog rod outer segments used by us have lost some calcium during the isolation procedure, the calcium content of these suspensions, as determined by atomic absorption spectroscopy (Table VI), is still very high, ca. 12 moles of calcium being present per mol rhodopsin, or 30 mM on total outer segment volume basis. This value is considerably higher than the 1-2 moles of calcium per mol rhodopsin, reported by Liebman (1974). His isolation procedure, however, takes considerably more time than ours en he employs a Ringer solution without ATP, which may lower the calcium content significantly (Table VI). It is noticeable that our binding studies, using an equilibrium dialysis technique, show that cattle rod outer segment membranes may bind up to 10.5 moles calcium per mol rhodopsin, which value comes close to the endogeneous calcium content measured by atomic absorption spectroscopy in frog rod outer segments. Therefore it seems likely that rod outer segments in <u>vivo</u> do indeed have a very high calcium content.

The calcium-transmitter hypothesis would require an effect of light upon the calcium distribution in rod outer segments, viz. a light-induced decrease in calcium content of rod discs. Technically this might be difficult to prove: not only must the isolated outer segments retain their excitation mechanism, or at least the first steps, but the

proposed calcium shift might be very small (cf. Cone, 1973; Yoshikami and Hagins, 1973). This technical difficulty may explain the fact that only a few reports have been published on this topic since the first publication of the hypothesis (Yoskikami and Hagins, 1971).

While Bownds et al (1971) and Neufeld et al (1972) only find an ATP dependent calcium binding by photoreceptor membranes, which is not affected by light, three reports have recently been presented which claim a decrease in calcium content of rod discs upon illumination (Hemminki, 1975; Szuts and Cone, 1974; Mason et al, 1974b).

Mason et al (1974b) claim to show a light-induced calcium release from sonicated bovine disc membranes. Illumination of a suspension of sonicated photoreceptors in an EGTA-containing buffer does indeed yield a calcium efflux. However, no experiment is shown in which the EGTA-containing buffer is added to the outer segments in the dark, which in itself could very well cause a considerable calcium release. The absence of this experiment makes their results rather inconclusive.

The results reported in an abstract by Szuts and Cone (1974) resemble in some respects those described in chapter 3. We have fragmented the frog outer segments by a lysis procedure, which yields a particulate fraction retaining 46% of the original calcium content (Table VIII). This calcium fraction is responsive to light, i.e. illumination of the outer segment suspension, either before lysis or after lysis followed by resuspension in an ATP-containing isotonic solution, decreases significantly the calcium content of the particulate material (Table X). This shift seems rather specific for calcium ions, since the distribution of magnesium ions is not affected by light. The size of the effect is the same, whether 85% or 15% of the rhodopsin is bleached (Table XI). It can be calculated (cf. section 3.4.4) that calcium fluxes of this magnitude in <u>vivo</u> should be able to close a sufficient number of sodium channels in the outer membrane to explain the effects of light on the dark current.

The continuation of this type of experiments is hampered by the

fact that only outer segment suspensions obtained from Rana esculenta, and then only from certain groups of frogs, would yield this light effect in reproducible fashion. Similar difficulties have been encountered by Szuts and Cone. They find a light-induced release of calcium ions from outer segment fragments, though at lower bleaching percentages (0.01-1%; Szuts and Cone, 1974). However, this effect appears to be non-reproducible too (personal communication). A careful investigation of storage and feeding conditions of the frogs is apparently necessary to find out why only certain species and groups of frogs show the desired effect. If it should indeed be possible to obtain frog rod outer segment suspensions, which react reproducibly upon illumination with a change in calcium distribution, the lysis method will in our opinion yield a useful system for investigating the factors controlling the light-induced calcium release. Obviously further evidence is then needed to prove the physiological significance of the phenomenon, first by examining the effect of rhodopsin bleaching percentages lower than 15% and consequently by illumination with light of various wavelengths, to obtain an 'action spectrum' of the calcium release.

There are indications that the calcium released by illumination is bound to the disc membranes rather than stored inside the intradiscal space (section 3.4.4). It appears from the equilibrium dialysis experiments (chapter 4) that disc membranes do indeed contain rather specific binding sites for calcium (Table XII). Two classes of binding sites are present. High-affinity sites with an association constant of $1.2 \times 10^4\ M^{-1}$ and accomodating 20 nmoles calcium per mg membrane protein (approximately 1 mole calcium per mole rhodopsin) and low-affinity sites with an association constant of $2.4 \times 10^3\ M^{-1}$, which are able to accomodate 200 nmoles calcium per mg protein (or 9.4 moles calcium per mole rhodopsin). Since the calcium binding has not been investigated at calcium concentrations below 10^{-5}M, it would appear useful to apply EGTA-calcium buffers, which can lower the free calcium concentration

considerably further, to investigate whether a small number of sites with still higher affinity for calcium may be present.

Although the rod outer segment suspensions used for the equilibrium dialysis experiments are obtained from cattle retinas after a relatively lengthy (ca. 5 h) isolation procedure, illumination still causes a significant decrease in the binding capacity. This again indicates that release of calcium from specific binding sites on the disc membrane, is an integral part of the visual excitation process. The same type of experiments, but using another method for measuring calcium binding, have been reported by Hemminki (1975).

The re-uptake of calcium, obviously necessary during dark-adaptation, might be mediated by phosphorylation. Light induces phosphorylation of opsin (Kühn et al, 1973; Weller et al, 1975). The time course of this reaction is such, that it is probable that the phosphorylation is part of the dark-adaptation process. We have found indications (Table XIII) that the additional phosphate groups possibly are able to bind calcium. Thus it appears possible that phosphorylation of rhodopsin is part of the process which binds calcium ions back to the disc membranes after they have been released by illumination. A further question to be examined is whether the calcium is merely bound to the photoreceptor membrane or is also transported across it into the intradiscal space.

To summarize, the results from chapter 3 and 4 support the hypothesis that calcium ions are functioning as transmitters between rod discs and rod outer membranes. Fig. 33 shows the present concept of the excitation chain in vertebrate photoreceptor cells. However, much is still to be learnt about the detailed molecular mechanisms underlying this receptor process.

7.4. MODEL EXPERIMENTS

Transport of sodium and calcium ions through photoreceptor membranes probably is an essential link in visual excitation (fig. 33).

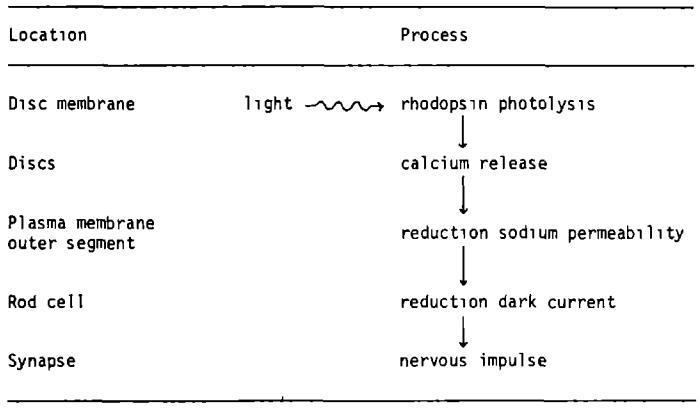


Fig. 33. Present scheme for the regulation of rod excitation.

Since phospholipid bilayers are considered to play an important part in the structure and function of biological membranes, we have investigated the passive leakage of sodium and calcium ions through phospholipid model membranes (chapter 5 and 6). Liposomes have been formed from phosphatidylcholine, phosphatidylcholine-phosphatidylserine mixtures, retinal lipid extracts and rod outer segment extracts, and the efflux of sequestered ^{22}Na and ^{45}Ca from these vesicles has been examined.

It appears that the lipid bilayers, composed of rod outer segment lipids, are more permeable to both calcium and sodium than the retinal lipid bilayers (Fig. 31), the difference being larger in the case of sodium ions. Assuming that the phospholipid composition of whole retina (Anderson, 1970) is about equal to that from isolated rod outer segments (Daemen, 1973), another factor than the phospholipid composition must be responsible for this difference. This is in our opinion the special fatty acid composition of the rod outer segment membranes, which has a highly unsaturated character. The percentage docosahexaenoic acid in our extracts (48%) is higher than previously reported (cf. Daemen, 1973). This may be due to the use of improved procedures for outer segment

isolation, which could minimize loss of double bonds.

It appears possible that the high degree of unsaturation in the lipid core of the outer segment membranes is an important factor in the visual excitation process by conferring a high permeability to the ions concerned.

The sodium leakage from rod outer segment lipid liposomes is in our experiments not affected by the presence of calcium ions (Table XVI). Since it is postulated that after illumination calcium ions close part of the sodium channels in the outer segment outer membrane, this may probably mean that this calcium effect is mediated by the protein part of the membrane.

While the leakage of calcium ions from rod outer segment lipid liposomes is faster than from retinal lipid liposomes, the permeability of these liposomes is still considerably lower than those of natural membranes (cf. Martonosi et al, 1974). Therefore, it might be expected that the proteins of the outer segment membrane are responsible for a great increase in permeability, like the ionophoric compounds A23187 and X537A greatly enhance the permeability of retinal lipid liposomes for calcium ions (Fig. 27).

The examination of the ionophore-induced increase in calcium leakage from retinal lipid liposomes has yielded some interesting results about the permeant ionophore-calcium complexes. At high ionophore concentrations a 1:2 Ca^{2+}-ionophore complex exists for both ionophores, while at low ionophore concentrations two different complexes appear to exist, a 1:1 Ca-X537A complex and a 2:1 Ca-A23187 complex.

It now appears possible to reconstitute rod outer segment membranes from rhodopsin, purified by affinity chromatography with concanavalin A, and lipid material, essentially by means of the method described by Hong and Hubbell (1972, 1973). This technique offers great possibilities for investigating the role of rhodopsin in determining the permeability characteristics of rod outer segment membranes. It will be very

interesting to see whether the incorporation of rhodopsin into liposomes of different lipid composition indeed enhances their permeability for calcium ions and confers a 'calcium-sensitivity' to their sodium permeability.

Er is relatief weinig bekend over het molekulaire mechanisme dat ten grondslag ligt aan de visuele excitatie, het proces dat een lichtprikkel omzet in een zenuwimpuls. De eerste stap in dit proces is de door licht geinduceerde isomerisatie van de chromofore groep van rhodopsine, 11-cis retinaldehyde, naar all-trans retinaldehyde (Wald, 1968). Uit de elektrofysiologie komen sterke aanwijzingen dat een volgende stap in dit proces een permeabiliteitsverlaging voor natrium ionen van t staafjes buitenmembraan is. Aangezien het rhodopsine grotendeels gelokaliseerd is in de zakjesmembranen, die struktureel niét verbonden zijn met het celmembraan (Fig. 2), levert dit het volgende probleem op: hoe kan een isomerisatie in het rhodopsine op het zakjesmembraan leiden tot een permeabiliteitsverandering van het buitenmembraan ? De meest logische beantwoording van deze vraag lijkt te liggen in het postuleren van een transmitter, een molekuul dat onder invloed van belichting uit de zakjes naar het buitenmembraan diffundeert.

Yoshikami en Hagins (1971; vgl. Hagins, 1972) hebben, op grond van elektrofysiologische experimenten, in hun model deze transmitter funktie toegekend aan het calcium ion (Fig. 9). Een andere mogelijkheid zou het cAMP zijn (Bitensky et al, 1971, 1972a,b), een verbinding die als 'second messenger' werkzaam is in vele hormonaal gestimuleerde processen.

Dit proefschrift beschrijft experimenten die er grotendeels op gericht zijn de calcium-transmitter hypothese te testen. Naast direkt onderzoek naar calcium niveaus in kikkerstaafjes (hoofdstuk 3) en calcium binding aan runderstaafjes membranen (hoofdstuk 4), beschrijven twee hoofdstukken (5 en 6) experimenten aan calcium en natrium diffusie door modelmembranen. Bovendien is de aanwezigheid van het cAMP vormende enzym, adenylaat cyclase, in fotoreceptoren onderzocht (hoofdstuk 2).

Hoofdstuk 1 geeft een overzicht van onze huidige morfologische en (bio)chemische kennis van het visuele systeem der vertebraten, waarbij de meeste nadruk is gelegd op het aspekt der visuele excitatie.

Door Bitensky et al is gerapporteerd dat staafjes suspensies een extreem hoge adenylaat cyclase aktiviteit bevatten, die vrijwel volledig kan worden geremd door belichting. In hoofdstuk 2 zijn onze pogingen beschreven om deze aktiviteiten te verifiëren. Het blijkt dat de werkelijke aktiviteit in zowel kikker- als runderstaafjes een faktor 100 lager is (0.2-0.5 nmol cAMP/mg eiwit per 10 min) dan beschreven door Bitensky's groep. Bleking van 80% van het aanwezige rhodopsine remt deze aktiviteit slechts voor 40-50%. Bovendien wordt aangetoond dat zich, bij fraktionering van een runder retinahomogenaat op een sukrose dichtheidsgradiënt, vlak onder de rhodopsine band een fraktie bevindt met een 10 x hogere specifieke adenylaat cyclase aktiviteit. Het blijkt dat deze enzym aktiviteit, toegevoegd aan de rhodopsine bevattende fraktie, eveneens door belichting kan worden geremd, hetgeen een aanwijzing is dat licht uit de staafjes een substantie (calcium ?) losmaakt die in staat is adenylaat cyclase aktiviteiten te remmen. Uit deze resultaten kan, mede op grond van andere inmiddels verschenen publikaties, worden gekonkludeerd dat het zeer twijfelachtig is of de adenylaat cyclase aktiviteit in <u>vivo</u> in de staafjes aanwezig is en dat cAMP naar alle waarschijnlijkheid geen rol speelt bij de visuele excitatie.

Hoofdstuk 3 beschrijft experimenten aan kikkerstaafjes waarin de endogene calcium niveaus zijn gemeten met behulp van atomaire absorptie spektrofotometrie. De suspensies van geïsoleerde kikkerstaafjes bevatten een hoog gehalte aan calcium, 12.4 nmol calcium per mol rhodopsine, hetgeen neerkomt op een concentratie van 30 mM, berekend op het totale staafjes volume. Lysis van de staafjes in 4 volumina water levert na centrifugatie een oplosbare en een membraan fraktie op, waarbij in de membraan fraktie 46% van de oorspronkelijke hoeveelheid calcium achterblijft. Deze fraktie is licht-gevoelig, dat wil zeggen, belichting van staafjes, hetzij voor lysis hetzij na lysis en resuspenderen in een ATP-bevattende isotone buffer oplossing, veroorzaakt een signifikant verlies van calcium uit deze fraktie. Bij vermindering van de

belichtingssterkte (verlaging van het blekingspercentage van rhodopsine van 85% naar 15%) blijft het effekt even groot. Experimenten met een calcium ionofoor en met EGTA- en EDTA-bevattende buffers suggereren dat het calcium dat vrijkomt bij belichting gebonden is geweest aan het zakjesmembraan en niet afkomstig is uit een oplosbare, door een membraan omgeven, fraktie.

De calcium bindende capaciteit van runderstaafjes membranen is onderozcht met behulp van een evenwichts dialyse techniek (hoofdstuk 4). Het feit dat, zelfs in aanwezigheid van een overmaat natrium en magnesium ionen, deze membranen calcium binden, duidt op het bestaan van calcium-specifieke bindingsplaatsen. De hoeveelheid calcium die gebonden wordt is sterk afhankelijk van de calcium concentratie in het medium. Uit een Scatchard plot blijkt dat er twee soorten bindingsplaatsen op het membraan aanwezig zijn: plaatsen met een hoge affiniteit (associatiekonstante 1.2×10^4 M^{-1}; bindingscapaciteit 20 nmol calcium per mg membraaneiwit of ca. 1 mol calcium per mol rhodopsine) en plaatsen met een lagere affiniteit voor calcium (associatiekonstante 2.4×10^3 M^{-1}; bindingscapaciteit 200 nmol calcium per mg eiwit of 9.4 mol calcium per mol rhodopsine). Belichting blijkt de bindingscapaciteit van de membranen op signifikante wijze te kunnen beïnvloeden: belichte membranen binden minder calcium.

Deze resultaten ondersteunen, samen met die uit hoofdstuk 3, de hypothese dat calcium als transmitter fungeert in het visuele excitatie proces.

Licht blijkt, op een meer indirekte wijze, ook een tegengestelde (= verhogende) invloed op de calcium binding te kunnen hebben. Licht induceert fosforylering van opsine in aanwezigheid van ATP (vgl. Kühn et al, 1973; Weller et al, 1975). Mogelijk kunnen de extra ingevoerde fosfaatgroepen calcium binden (Tabel XIII), zodat de fosforylerings reaktie deel uitmaakt van het proces dat door licht losgemaakte calcium ionen weer terug bindt aan de zakjes.

In hoofdstuk 5 worden experimenten beschreven die de diffusie van calcium ionen uit liposomen betreffen. Liposomen zijn opgebouwd uit een centraal kompartiment dat omgeven is door een of meerdere (fosfo)lipide bilagen. Het is mogelijk om tijdens de bereiding van deze vesicles radioaktieve ionen in te sluiten en daarna de passieve lek van deze ionen te volgen met behulp van dialyse tegen een isotoop-vrij medium. De ionenlek wordt beschreven met behulp van een mathematisch model.

De liposomen zijn opgebouwd uit fosfatidylcholine, een fosfatidylcholine-fosfatidylserine (1:1) mengsel en uit retinalipiden en de calciumlek is bepaald als funktie van tijd, temperatuur en calcium concentratie. Liposomen opgebouwd uit retinalipiden verliezen het ingesloten ^{45}Ca zeer langzaam, ongeveer even snel als fosfatidylcholine liposomen, terwijl de liposomen opgebouwd uit fosfatidylcholine en fosfatidylserine (1:1) hun ^{45}Ca 20 x sneller verliezen.

Introduktie van de ionoforen X537A en A23187 verhoogt de calciumlek uit retina lipide liposomen in hoge mate, waarbij A23187 aanzienlijk effektiever (i.e. bij lagere concentraties) werkt dan X537A. Voor beide ionoforen is de calcium efflux snelheid bepaald in afhankelijkheid van de ionofoor concentratie. Uit de resulterende kromme kan worden gekonkludeerd dat de ionoforen op verschillende wijze calcium lijken te transporteren. Bij hoge ionofoor concentraties wordt weliswaar in beide gevallen een 1:2 Ca-ionofoor komplex gevormd, maar bij lagere ionofoor concentraties transporteert X537A calcium in de vorm van een 1:1 Ca-ionofoor komplex, terwijl A23187 een 2:1 Ca-ionofoor komplex vormt.

In hoofdstuk 6 is de efflux van ^{22}Na beschreven uit liposomen van verschillende samenstelling. Naast fosfatidylcholine en fosfatidylcholine-fosfatidylserine (4:1) liposomen is de natriumlek uit liposomen opgebouwd uit retina en staafjes lipiden vergeleken. Het blijkt dat staafjes lipide liposomen bijzonder permeabel zijn vergeleken met retina lipide liposomen: ze verliezen natrium 7-13 maal sneller (afhankelijk van het dialysemedium). Aangezien de fosfolipide samenstelling van beide

lipide extracten ongeveer gelijk is, is het waarschijnlijk dat het verschil in permeabiliteit veroorzaakt wordt door de hoge onverzadigingsgraad van de vetzuren in de staafjesmembranen. Bepaling van de vetzuursamenstelling toont inderdaad aan dat vooral het gehalte aan docosahexeenzuur (22:6) spektakulair verschilt: 48% in de extracten uit staafjes tegen 24% in het retina extract. Deze 48% is aanzienlijk hoger dan eerder gepubliceerde percentages (23-37%). De natriumlek uit de staafjes lipide liposomen blijkt niet te worden beïnvloed door de aanwezigheid van calcium ionen in het dialysemedium, zodat het waarschijnlijk is dat het proteine gedeelte van het membraan verantwoordelijk is voor het sluiten van de natrium-kanalen (door calcium ionen) na belichting.

Tenslotte worden in hoofdstuk 7 de belangrijkste resultaten samengevat en besproken in het licht van de recentelijk gepubliceerde literatuur. Verder worden enige suggesties gedaan voor toekomstige experimenten.

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Thijs Hendriks werd op 13 oktober 1946 geboren te Amersfoort, alwaar hij de lagere school en het plaatselijk lyceum (gymnasiumβ) doorliep. In 1964 werd begonnen met de studie scheikunde aan de Universiteit van Amsterdam, hetgeen in juni 1968 resulteerde in het behalen van het kandidaatsexamen S_2 en in juli 1971 werd afgesloten met het doktoraal-examen (hoofdvak: biochemie; bijvakken: plantenfysiologie en chemische mikrobiologie). Sinds 1 augustus 1971 is hij als wetenschappelijk medewerker verbonden aan de afdeling biochemie van de medische fakulteit van de Universiteit van Nijmegen, waar het hier beschreven promotie-onderzoek is uitgevoerd onder leiding van Dr. F.J.M. Daemen en Prof.Dr. S.L. Bonting.

STELLINGEN

1. Het feit dat in fotoreceptor suspensies enzymaktiviteiten voorkomen die gevoelig zijn voor licht, betekent niet zonder meer dat deze enzymen ook in vivo in de fotoreceptoren zijn gelokaliseerd.
   Dit proefschrift, hoofdstuk 2.

2. Evaluatie van de hypothese van Bitensky c.s., dat cAMP een belangrijke rol speelt in het mechanisme van de visuele excitatie, wordt ernstig belemmerd door hun publikaties.
   M.W.Bitensky, R.E.Gorman en W.H.Miller, Proc.Natl.Acad.Sci. 68 (1971) 561-562.
   W.H.Miller, R.E.Gorman en M.W.Bitensky, Science 174 (1971) 295-297.
   M.W.Bitensky, R.E.Gorman en W.H.Miller, Science 175 (1972) 1363-1364.

3. De konklusie van Mason c.s., dat staafjes zakjes membranen calcium ionen verliezen ten gevolge van lichtabsorptie, kan onmogelijk op grond van hun eigen experimenten worden getrokken.
   W.T.Mason, R.S.Fager en E.W.Abrahamson, Nature 247 (1974) 562-563.

4. Het is niet afdoende bewezen dat de door Shamoo en Myers geïsoleerde natrium ionofoor afkomstig is uit het (Na-K)-ATPase.
   A.E.Shamoo en M.Myers, J.Membrane Biol. 19 (1974) 163-178.

5. Gebruik van de (Na-K)-ATPase aktiviteit in de erythrocyt membranen is minder zinvol wanneer men de algemene eigenschappen van dit enzym wil bestuderen.

6. Voor het verklaren van een vertraagd metabolisme van geneesmiddelen optredend bij leverziekten, zijn een gehaltebepaling van cytochroom P-450 en het verrichten van leverfunktietests alleen niet voldoende, maar dient het metabolisme eveneens met behulp van gericht gekozen teststoffen als parameters te worden gekontroleerd.

   E.F.Hvidberg, P.B.Andreasen en L.Ranek, Clin.Pharmacol.Therapeut. 15 (1973) 171-177.

   U.Klotz, G.R.Avant, A.Hoyumpa, S.Schenker en G.R.Wilkinson, J.Clin.Invest. 55 (1975) 347-359.

7. De door Prada-Alcala c.s. gemaakte vooronderstelling, dat het elektrisch stimuleren van een nucleus in de hersenen eenzelfde resultaat heeft als de stimulering van een zenuwbaan die deze nucleus innerveert, is onjuist.

   R.A.Prada-Alcala, E.W.Kent en L.D.Reid, Brain Res. 84 (1975) 531-540.

8. De regulering van de aanpassing van de bouwcapaciteit aan de dalende bouwproduktie in de komende jaren, dient zoveel als mogelijk te geschieden door prijsvorming op de openbare bouwmarkt. Hierdoor verbetert de kennis van het bouwen aan de zijde van de opdrachtgever en vindt de meest aanvaardbare aanpassing plaats van de capaciteit van het uitvoerend bouwbedrijf.

9. Het verdient aanbeveling om op zeer korte termijn in elke academische studie plaats in te ruimen voor het onderdeel vrijetijdsbesteding.

10. Ter bestrijding van de spelverruwing in het Nederlandse voetbal dient op korte termijn een 'van Hanegem' trofee te worden ingesteld voor dié speler, die zijn carrière afsluit zonder ooit een officiële waarschuwing te hebben ontvangen.

Th. Hendriks
2 april 1975